Chem. Anal. (Warsaw), **52**, 645 (2007)

Simultaneous Determination of Sildenafil, Its N-Desmethyl Active Metabolite and Other Drugs in Human Urine by Gradient RP–HPLC Method

by Irena Baranowska[1,*], Piotr Markowski[1], Jacek Baranowski[2] and Jarosław Rycaj[3]

[1] Department of Analytical and General Chemistry, Silesian University of Technology, ul. M. Strzody 7, 44-100 Gliwice, Poland
[2] Department of Clinical Physiology, University Hospital, SE-581 85 Linköping, Sweden
[3] Department of Pediatric Cardiology, Silesian Centre of Heart Disease, Medical University of Katowice, ul. Szpitalna 2, 44-800 Zabrze, Poland

Keywords: Sildenafil; N-Desmethylsildenafil (UK–103,320); Drugs; Human urine; RP–HPLC

A new, rapid, sensitive and accurate gradient reversed-phase high-performance liquid chromatography technique for simultaneous separation and analysis of sildenafil citrate (SC), its N-desmethyl active metabolite – N-desmethylsildenafil (UK–103,320) in the presence of different drugs in human urine was developed. The analysed drugs were extracted from urine by liquid-liquid extraction. Effective RP–HPLC separation of the examined drugs was performed using a Merck LiChroCART® analytical column (Purospher® STAR RP–18 endcapped, 125 × 3 mm, particle size 5 μm) with a gradient mobile phase system and diode array or fluorescence detector. Linear ranges of detection for SC and UK–103,320 were found to be 0.03–8.5 μg mL^{-1} (r^2 = 0.9994) for both compounds. Linear ranges for other drugs (analgesic, antibiotic, diuretic and demulcent), which could exist in urine from patients treated with SC were also determined. Complete separation of all analytes was achieved below 25 min. The retention times for all studied analytes ranged from 4.76 to 18.84 min. The limits of detection and limits of quantification for both analysed compounds were calculated and recovery studies were also performed. The mean absolute recoveries of SC and UK–103,320 were > 94%. The new procedure was suitably validated and successfully applied for the analysis of SC, its active metabolite and other drugs in urine samples of patients with pulmonary hypertension.

* Corresponding author. E-mail: irena.baranowska@polsl.pl; Tel./Fax: +48 32 237 1205

Opracowano nową, szybką, czułą i dokładną gradientową technikę wysokosprawnej chromatografii cieczowej w odwróconym układzie faz do jednoczesnego rozdzielania i oznaczania cytrynianu sildenafilu (SC) i jego N-desmetylo aktywnego metabolitu – N-desmetylosildenafilu (UK–103,320) w obecności różnych leków w ludzkim moczu. Badane leki ekstrahowano z próbek moczu przy użyciu ekstrakcji ciecz-ciecz. Efektywne rozdzielanie badanych leków metodą RP–HPLC wykonywano z zastosowaniem kolumny analitycznej Merck LiChroCART® (Purospher® STAR RP–18 endcapped, 125 × 3 mm, średnica ziarna 5 µm) z gradientowym przepływem fazy ruchomej i detektorem z matrycą fotodiodową lub detektorem fluorescencyjnym. Liniowe zakresy detekcji SC i UK–103,320 mieściły się w przedziale 0.03–8.5 µg mL^{-1} (r^2 = 0.9994) dla obu związków. Wyznaczono również zakresy liniowości dla pozostałych leków (przeciwbólowe, antybiotyki, diuretyk i przeciwzapalne), które mogą współistnieć w moczu pacjentów leczonych SC. Całkowity rozdział wszystkich analitów osiągnięto w czasie poniżej 25 min. Czasy retencji dla wszystkich badanych analitów mieściły się w przedziale od 4.76 do 18.84 min. Wyznaczono granice wykrywalności i oznaczalności oraz odzyski wszystkich analizowanych związków. Wartości całkowitych odzysków dla SC i UK–103,320 były większe niż 94%. Nowa procedura została zwalidowana oraz z powodzeniem zastosowana do analizy SC, jego aktywnego metabolitu oraz innych leków w próbkach moczu pacjentów z nadciśnieniem płucnym.

There are a number of multicenter studies in progress now with the aim to evaluate the value of sildenafil citrate (SC) in the treatment of primary and secondary pulmonary hypertension. The aim of the treatment of pulmonary hypertension is to achieve a pulmonary vasodilatation. Pulmonary vasodilatation causes the decrease of pulmonary vascular resistance and increases cardiac index, which are the haemodynamic goals of the therapy when improved exercise tolerance is a clinical one. SC is a selective inhibitor of type 5 phosphodiesterase (PDE5), a phosphodiesterase isoenzyme that metabolizes cyclic guanosine-3'-5'-monophosphate (cGMP) which is a mediator of vasodilatation and is presented in smooth muscles of pulmonary vascular bed [1]. Therefore, SC increases cGMP within pulmonary vascular smooth muscle cells resulting in relaxation. In patients with pulmonary hypertension, this can lead to vasodilatation of the pulmonary vascular bed and, to a lesser degree vasodilatation in the systemic circulation. Pulmonary hypertensive patients have higher susceptibility to respiratory tract infections and heart failure. These are frequently treated with non-steroid, steroid anti-inflammatory drugs and antimicrobial agents mostly antibiotics and fungicidal drugs. The data shows the interactions of the drugs alone, and in combinations with SC metabolism is still very limited. In this study, by preparing a method of examining the number of drugs that can be found in many situations administered together with SC to people suffering from pulmonary hypertension and infection or heart failure the authors tried to help the researchers focused on drug interactions to find a useful tool for their studies. Therefore it is necessary to develop one chromatographic system which could provide simultaneous determination of concurring drugs in the shortest time.

The major circulating metabolite of SC, UK–103,320 results from piperazine N-desmethylation and has a 2.5 fold lower *in vitro* potency for PDE5 [2–4]. Quantification of SC and UK–103,320 is therefore essential during the evaluation of the parent drug. The simultaneous determination of SC and the metabolite UK–103,320 is also necessary in the pharmacokinetic study of SC. High-performance liquid chromatography (HPLC) using UV detector [5–7], gas chromatography–mass spectrometry [8], micellar electrokinetic chromatography [9], liquid chromatography-tandem mass spectrometry [3, 10] and adsorptive stripping square-wave voltammetry [4] methods have been reported for the analysis of SC and/or UK–103,320 in biological samples.

A procedure for the simultaneous determination of SC and UK–103,320 in human plasma using column switching was also reported [11]. These methods involve liquid-liquid extraction (LLE) [3], solid-phase extraction [4, 7, 9] or automated sequential trace enrichment of dialysates [6] as sample preparation procedures.

Imipenem (IMP) is a β-lactam antibiotic with a broad spectrum for microorganisms. Paracetamol (PAR) is a valuable non-steroidal inflammatory, analgesic and antipyretic drug in widespread use for pain management as antipyresis. Amikacin (AMK) is an aminoglycoside drug with a very broad antimicrobial spectrum extending from Gram-positive to Gram-negative bacilli. Vancomycin (VCM) is a glycopeptide antibiotic many Gram-positive bacterial infections including methicillin-resistant *Staphylococci epidermidis*. Cefazolin (CFZ) is a β-lactam antibiotic, which has potential anti-bacterial activity against a broad spectrum of microorganisms. Dipyrone (DPR) is an antipyretic, analgesic and anti-inflammatory drug. Fluconazole (FZ) is a synthetic triazole derivative antifungal agent; it has been shown to be effective against a wide range of systemic and superficial fungal infections, following both oral and intravenous administrations. Furosemide (FUR) is a potent diuretic drug currently used in clinical practice. Prednisolone (PRE) is a widely used synthetic glucocorticoid with reduced mineralocorticoid side effects compared to the physiological steroid hydrocortisone. Dexamethasone (DEX) is a glucocorticoid with strong anti-inflammatory and anti-allergic activity. Ketoprofen (KET), an aryl propanoic acid derivate, is a potent non-steroidal anti-inflammatory agent which has also analgesic and antipyretic properties [12].

Different analytical methods have been applied for the determination of the above-mentioned drugs, including HPLC techniques. HPLC with UV detection was found to be the technique of choice for many workers and was developed using LC–UV alone. There have been many HPLC methods describing IMP [13, 26–29], PAR [14, 30–38], AMK [15, 39–41], VCM [16–18, 42–49], CFZ [19, 50], DPR [20, 51], FZ [21, 52–55], FUR [22, 56–61], PRE [23, 62–72], DEX [24, 73–76] and KET [25, 77–84] analysis in biological fluids or pharmaceuticals. Simultaneous analysis of antibiotics from cephalosporins group in human serum was carried out [50]. Simultaneous separation and determination of DPR, PAR and caffeine in pharmaceutical formulation has been described [51].

In this work, a new RP–HPLC system and LLE procedure for sample preparation and determination of SC, UK–103,320 in the presence of selected drugs have been elaborated and applied for the analysis of urine of patients treated with SC. There is no analytical method for the simultaneous analysis of SC, UK–103,320 in the presence of eleven drugs belonging to four different groups in human urine samples described in scientific literature.

Developed system has been used in the marking of examined compounds in human urine samples. The aim of the study was to evaluate an analytical method with the optimum parameters for simultaneous separation and determination of SC, UK–103,320, IMP, PAR, AMK, VCM, CFZ, DPR, FZ, FUR, PRE, DEX and KET in human urine samples. The combinations of analysed drugs are in agreement to the ones most often used in the ward and most frequently found together in urine samples. Separate passing of above-mentioned drugs to patients (chromatographic system enables) gives the possibility to differentiate them in human urine samples. This work was performed to develop a relatively simple HPLC method based on reversed-phase, gradient elution, diode array or fluorescence detection. This study was also conducted to evaluate one RP–HPLC–FL derivatization method for quantitative determination of AMK. The method has been validated under stringent criteria.

EXPERIMENTAL

Chemicals and reagents

Sildenafil citrate (70.7% activity) {IUPAC name: 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)phenylsulfonyl]-4-methylpiperazine citrate} and its N-desmethyl metabolite (UK–103,320) {IUPAC name: 1-[4-ethoxy-3-(6,7-dihydro-1-methyl-7-oxo-3-propyl-1H-pyrazolo[4,3-d]pyrimidin-5-yl)phenylsulfonyl]piperazine} were obtained from Pfizer Pharmaceuticals (New York, USA). Ketoprofen {IUPAC name: 2-(3-benzoylphenyl)propanoic acid}, dexamethasone {IUPAC name: 9-fluoro-11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13,16-trimethyl-6,7,8,11,12,14,15,16-octahydrocyclopenta[a]phenanthren-3-one}, prednisolone {IUPAC name: 11,17-dihydroxy-17-(2-hydroxyacetyl)-10,13-dimethyl-6,7,8,9,10,11,12,13,14,15,16,17-dodecahydrocyclopenta[a]phenanthren-3-one}, furosemide {IUPAC name: 4-chloro-2-(2-furylmethylamino)-5-sulfamoyl-benzoic acid}, vancomycin hydrochloride {IUPAC name: not available} and cefazolin sodium salt {IUPAC name: sodium 4-[(5-methyl-1,3,4-thiadiazol-2-yl)sulfanylmethyl]-7-oxo-8-(2-tetrazol-1-ylacetyl)amino-2-thia-6-azabicyclo[4.2.0]oct-4-ene-5-carboxylate} (purity, 97–99.9%) were purchased from Sigma–Aldrich (Schnelldorf, Germany). The stock solution contained fluconazole {IUPAC name: 2-(2,4-difluorophenyl)-1,3-bis(1,2,4-triazol-1-yl)propan-2-ol} at a concentration of 2 mg mL^{-1} (Diflucan®) was obtained from Pfizer (Amboise, France). Paracetamol {IUPAC name: N-(4-hydroxyphenyl)acetamide}, imipenem {IUPAC name: 3-[2-(aminomethylideneamino)ethylsulfanyl]-6-(1-hydroxyethyl)-7-oxo-1-azabicyclo[3.2.0]hept-2-ene-2-carboxylic acid}, amikacin dihydrate {IUPAC name: 4-amino-N-[5-amino-2-[4-amino-3,5-dihydroxy-6-(hydroxymethyl)tetrahydropyran-2-yl]oxy-4-[6-(aminomethyl)-3,4,5-trihydroxy-tetrahydropyran-2-yl]oxy-3-hydroxy-cyclohexyl]-2-hydroxy-butanamide dihydrate} (purity, 97–99.5%), 3-mercaptopropionic acid

(3MPA) (purity, ≥ 99%) and o-phthaldialdehyde (OPA) (purity, ≥ 98.5%) were kindly provided by Fluka (Darmstadt, Germany). Dipyrone monohydrate {IUPAC name: sodium [(1,5-dimethyl-3-oxo-2-phenyl-pyrazol-4-yl)-methyl-amino]methanesulfonate hydrate} (purity, ≥ 99%) was bought from Riedel-de Haën (Seelze, Germany). Chloroform and ethyl acetate, all of analytical grade, were purchased from POCH S.A. (Gliwice, Poland). Acetonitrile, water, buffers (boric acid/potassium chloride/sodium hydroxide, pH 9.00), (acetic acid/sodium acetate, pH 4.66), dichloromethane and trifluoroacetic acid (an acidic mobile phase additive) for HPLC analysis (HPLC grade), were bought from Merck (Darmstadt, Germany). The chemical structures of SC and UK–103,320 are shown in Figure 1.

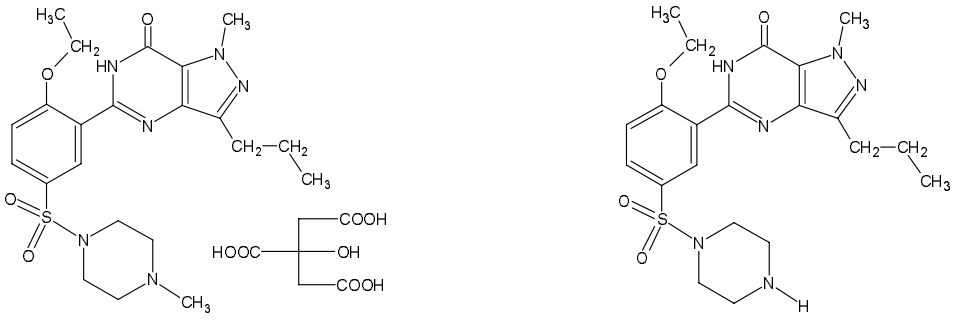

Figure 1. Chemical structures of SC and UK–103,320

Equipment and instrumentation

Reversed-phase HPLC analyses were carried out using a Merck Hitachi (Darmstadt, Germany) liquid chromatographic system consisting of Model L–6200A Intelligent Pump with a dynamic mixing chamber, a Model L–4500A diode array (DAD) or Model L–7480 fluorescence (FL) detector. Samples were injec-ted with a 7161 Rheodyne injector system with a 20 µL sample loop. Analytes were separated on a Merck LiChroCART® analytical column (Purospher® STAR RP–18 endcapped, 125 × 3 mm, particle size 5 µm) (Darmstadt, Germany) equipped with a Merck LiChroCART® pre-column (LiChrospher® 100 RP–18, 4 × 4 mm, particle size 5 µm) (Darmstadt, Germany). A centrifuge HERMLE Z 323K (Gosheim, Germany) was utilized to centrifuge urine samples. Programmes and calculations were performed by a HPLC System Manager HSM D–7000, version 2.1 (Merck Hitachi).

Chromatographic conditions

Experiments were performed at ambient laboratory temperature (*ca* 22°C). The effective separation of examined drugs (SC, UK–103,320, IMP, PAR, AMK, VCM, CFZ, DPR, FZ, FUR, PRE, DEX and KET) were carried out using a gradient elution technique. The mobile phase was comprised of various proportions of acetonitrile, buffer (acetic acid–sodium acetate, pH 4.66) and 0.05% trifluoroacetic acid in water. The effluent from the column was monitored by DAD detector in the range of 200–450 nm. AMK after derivatization with o-phthaldialdehyde–3-mercaptopropionic acid (OPA–3MPA) reagent was quan-

tified using the fluorescence signal. The wavelengths of fluorescence detector were set at 355 and 415 nm for excitation and emission, respectively. The analysed compounds were baseline separated under the given chromatographic conditions. Under these experimental conditions analysed analytes were eluted in less than 25 min per sample. After the run was completed, the column reequilibration time was 10 min. The peak area was used for the quantification of SC, UK–103,320, IMP, PAR, AMK, VCM, CFZ, DPR, FZ, FUR, PRE, DEX and KET.

Human urine samples

Human urine was obtained from pooled samples collected from healthy volunteers. This urine was used for construction of the standard curves, and for the validation of the method. Seven human urine samples from patients treated with SC because of pulmonary hypertension were obtained from Silesian Centre of Heart Disease, Medical University in Katowice (Poland). Human urine samples after drugs administration were collected during 24 h. These urine samples were analysed immediately or they were stored at –18°C until analysis. The thawing was allowed at room temperature before processing of the urine sample. Samples were analysed by RP–HPLC as described further.

Preparation of solutions

Stock solutions. Stock solution of sildenafil citrate was prepared by dissolving 14.14 mg (equivalent to 10 mg sildenafil) in 10 mL of a mixture of water–methanol (1:1, v/v). Separate stock solutions of the remaining analysed drugs (except FZ) were prepared by dissolving 10 mg of each drug in 10 mL of a mixture of water–methanol (1:1, v/v). The FZ stock solution was prepared by diluting 5 mL solution of FZ (2 mg mL^{-1}) in 5 mL of a mixture of water–methanol (1:1, v/v). Stock solutions were prepared at the beginning of the study and were stored at 4°C. All prepared stock solutions were stable for at least one month.

Standard solutions. Individual standard solutions were obtained by diluting stock solutions with water. Mixed standard solutions containing investigated compounds were prepared by mixing appropriate amount of individual standard solutions and diluting these mixtures with water. The individual and mixed standard solutions were stable for at least three weeks at 4°C.

Working solutions. Working solutions of the analysed drugs were prepared from the individual or combined standard solutions by suitable dilutions in human drug-free urine in a 10 mL volumetric flasks. These urine samples spiked of examined drugs at ten different concentrations ranged from 0.02 to 50 μg mL^{-1} were used for preparation of the calibration curves. The stability of the working solutions at 4°C was established up to five days without any significant decrease in examined drugs concentrations.

Derivatization procedure for standard solutions of AMK

AMK was derivatized with OPA–3MPA reagent [85] to form an AMK–OPA–3MPA adduct. A OPA–3MPA reagent was prepared by dissolving 40 mg OPA in 0.8 mL methanol, followed by addition of 7.2 mL of a buffer (boric acid–potassium chloride–sodium hydroxide, pH 9.00) and 40 μL 3MPA. A derivatization of AMK was performed by adding 20 μL of the derivatizing solution OPA–3MPA to 50 μL of standard solutions of AMK and vortex-mixing for 1 min. The pre-injection reaction time was 30 min at room temperature before the injection 20 μL of this solution into the RP–HPLC–FL system.

Samples preparation

Urine sample preparation and extraction method are described below step by step. The preparation of urine samples was by LLE. 3.0 mL of human urine sample was transferred to a volumetric flask and buffer (boric acid–potassium chloride–sodium hydroxide, pH 9.00) was used for adjusting the required pH (*i.e.*, pH 7). After vortex-mixing thoroughly for 1 min, the sample was transferred to a centrifuge tube and extracted with 5 mL mixture of ethyl acetate–dichloromethane–chloroform (45:35:20, v/v/v) by vortex--mixing for 5 min and centrifuged at 5500 rpm for 10 min at room temperature. Next, the aqueous layer was separated from the organic layer. The aqueous layer was discarded and the organic layer was evaporated to dryness under the stream of nitrogen at room temperature. The dried residue was dissolved with 100 µL of mixture acetonitrile–buffer (pH 9.00) (60:40, v/v). Twenty microliters of the sample was then injected into the RP–HPLC–DAD system for analysis. This method includes derivatization of AMK in urine samples prior to RP–HPLC determination using a system equipped with a FL detector. The previously prepared urine sample (50 µL) was mixed with OPA–3MPA reagent (20 µL) and vortex-mixed for 1 min. The pre-injection reaction mixture was than left for 30 min at room temperature and then 20 µL of this solution was injected into the RP–HPLC–FL system. Urine samples spiked with standards were treated with the same procedure. Concentrations of analysed compounds were determined by calculation of the peak area and interpolation of the respective calibration curve.

Calibration curves

Calibration curves ($y = ax + b$) of researched drugs were prepared by spiking appropriate amount of the combined standard solutions (ten different concentrations) into drug-free human urine. Calibration curves as peak area ratios *vs* standard concentrations were obtained with the use of a least-squares linear regression method.

The regression line was used to calculate concentrations of all compounds in the urine samples based on the peak area ratio. The peak area of each drug was plotted against the concentration to obtain the calibration graph. Detectors response in the corresponding calibration curves was linear. The sample pretreatment procedure was identical to the one described above.

Precision, accuracy and specificity

Precision, expressed as a coefficient of variation (C.V.) was determined by back-calculation of concentrations from the respective calibration curves. Known amounts of studied drug standard solutions were added to known amounts of real urine samples, which had already been analysed. The analytical precision of this method depends not only on the reproducibility of the isolation but also on the reproducibility of the derivatization reaction.

The method accuracy was evaluated by calculating the difference between the spiked sample peak area ratios and the original sample peak area ratios, then comparing these differences with the peak areas obtained by injecting standard solutions having the same concentration as the sample spiked. Accuracy, expressed as the percent error, was measured by determining the concentration of drugs measured in each sample relative to the known amount of each drugs added.

Specificity of the new RP–HPLC method was established to test matrix influence between different human urine samples from hospitalized patients.

Extraction yield and absolute recovery

Standard solutions of drugs were added to 3 mL of blank human urine, and then the samples were extracted according to the procedure described above, to obtain extraction yield values. The determination of the recovery rates was carried out from spiked human drug-free urine samples. Spiked urine samples were extracted and analysed for each concentration in ten replicates.

The absolute recovery of each analyte was estimated by comparing peak areas of each analyte in the extracted samples with those obtained from direct injection of the non extracted (standard) compounds. Extraction experiments were first performed using standard solutions, and then the procedure was checked with human urine samples. The absolute recovery was calculated for each analyte.

RESULTS AND DISCUSSION

Development of RP–HPLC method

Other drugs (which are commonly taken by patients suffering from primary pulmonary hypertension or Eisenmenger's syndrome) were taken into consideration as a possible cause of interference to this new analytical method. Chromatographic system, elaborated by the authors [85], for determination of eleven drugs (IMP, PAR, AMK,VCM, CFZ, DPR, FZ, FUR, PRE, DEX and KET) was not suitable for analysis of urine coming from patients treated with SC in view of interference SC and its UK–103,320 metabolite with these drugs. Therefore there was developed a new system acting for simultaneous determination of SC and its UK–103,320 metabolite in the presence of other drugs in urine coming from patients treated with SC suffering from pulmonary hypertension.

We have also studied optimization of the proposed procedure and examined conditions which could affect the results. The selection of suitable mobile phase relied as well on choosing buffer with adequate pH, which could enable separation SC and its metabolite. The mobile phase was chosen after several trials with acetonitrile, 0.05% trifluoroacetic acid in water and buffer solutions in various proportions and at different pH values. Applications of gradient with growing elution force let separate compounds with lower polarity and reduce analyse time. The optimized gradient effluent conditions for analysis of thirteen drugs were obtained. The best resolution of investigated drugs was obtained using as a mobile phase a mixture of acetonitrile (solvent A), buffer (acetic acid–sodium acetate, pH 4.66) (solvent B) and 0.05% trifluoroacetic acid in water (solvent C) with a solvent gradient. The gradient programme of the optimum elution profile and the mobile phase flow rate are presented in Table 1. This gradient programme was selected to achieve maximum separation and sensitivity.

Table 1. Optimum gradient elution profile for separation of SC, UK–103,320 in the presence of different analytes in human urine; solvent A: acetonitrile; solvent B: buffer (acetic acid/sodium acetate, pH 4.66); solvent C: 0.05% trifluoroacetic acid in water

	Solvent			
Time, min	A %	B %	C %	Flow rate, mL min^{-1}
0	3	96	1	1.0
10	35	64	1	1.1
20	45	54	1	1.2
25	50	49	1	1.3

The new method was tested in model samples, and afterwards in urine samples for a final target application. A satisfactory separation of all investigated analytes from biological endogenous components in human urine was obtained. Drugs of interest were isolated using a LLE with a mixture of ethyl acetate–dichloromethane–chloroform (45:35:20, v/v/v) and separations were obtained using a reversed-phase column under gradient conditions with ultraviolet or fluorescence detection. LLE was used because of its high efficiency, selectivity and simplicity.

Determination of SC, UK–103,320 and other drugs, with the use of the described chromatographic system let us determine both the analytes simultaneously, qualitatively and quantitatively, within 25 min. Separation of the analysed drugs was performed by a Merck LiChroCART® analytical column protected with a Merck LiChroCART® pre-column.

A typical chromatogram of the mixed standards of all drugs, except AMK is shown in Figure 2A. The average retention times for SC, UK–103,320, IMP, PAR, VCM, CFZ, DPR, FZ, FUR, PRE, DEX and KET were 17.68, 16.97, 4.71, 5.91, 7.81, 9.40, 10.59, 11.83, 14.17, 15.12, 16.02 and 18.76 min, respectively. Typical chromatograms of an extract of drug-free sample registered with using DAD and FL detectors are shown in Figures 2B and 3A, respectively.

The extraction and RP–HPLC procedures for the determination of SC, UK–103,320 and eleven drugs in human urine samples were set following the most desirable chromatographic and detection conditions (Figs. 2C and 3B). These examined drugs were separated, with good peak resolution, sharpness and symmetry.

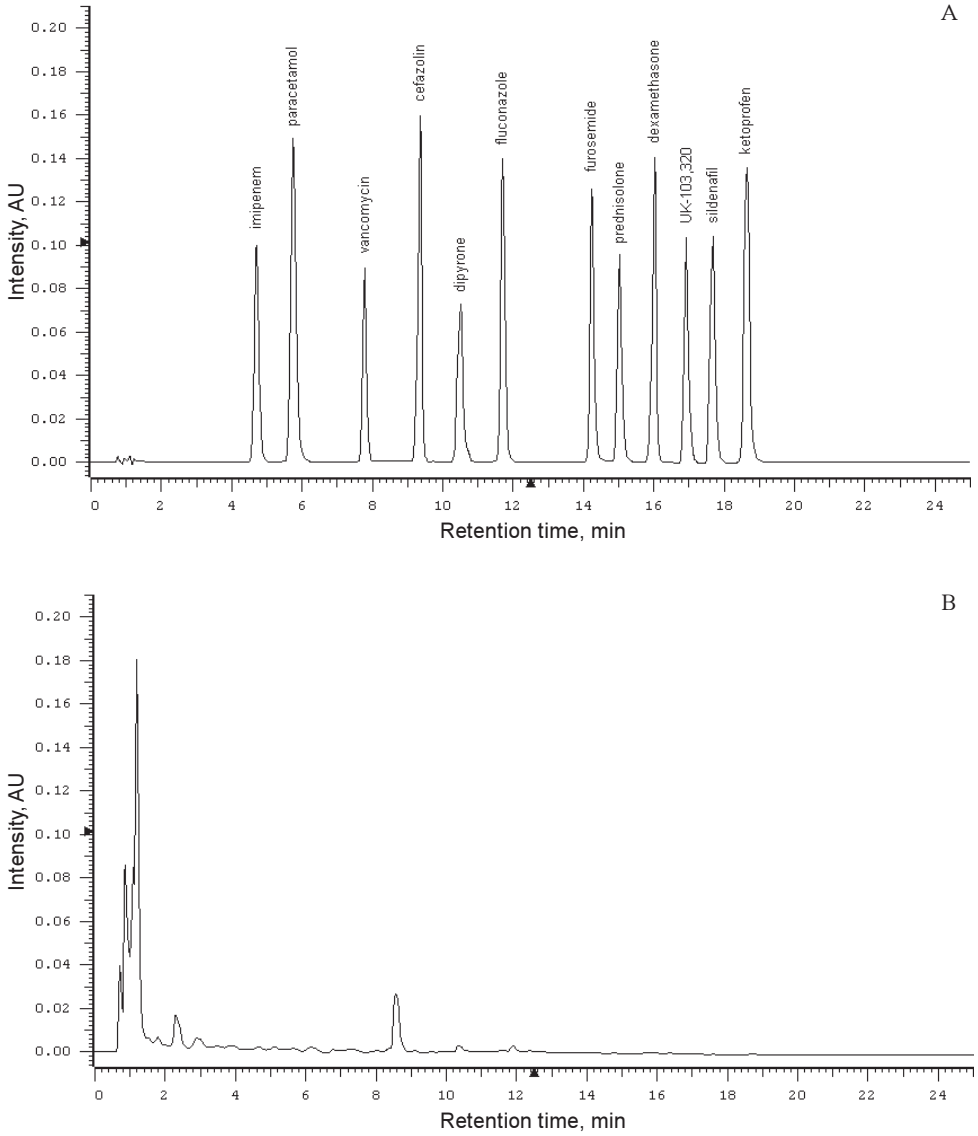

Figure 2. Representative RP–HPLC–DAD chromatograms of mixed standards containing: 0.17 μg of sildenafil, 0.17 μg of UK–103,320, 0.20 μg of imipenem, 0.30 μg of paracetamol, 0.16 μg of vancomycin, 0.30 μg of cefazolin, 0.30 μg of dipyrone, 0.40 μg of fluconazole, 0.24 μg of furosemide, 0.16 μg of prednisolone, 0.30 μg of dexamethasone and 0.30 μg of ketoprofen (A), sample prepared from a blank human urine (B), a calibration sample from a spiked human urine (C) and a patient urine sample (D). Detections were performed at 231 nm (for SC and UK–103,320), 300 nm (IMP), 243 nm (PAR), 210 nm (VCM and FZ), 274 nm (CFZ), 259 nm (DPR), 234 nm (FUR), 242 nm (PRE and DEX) and 254 nm (KET). Chromatographic conditions are described in text. (Continuation on the next page)

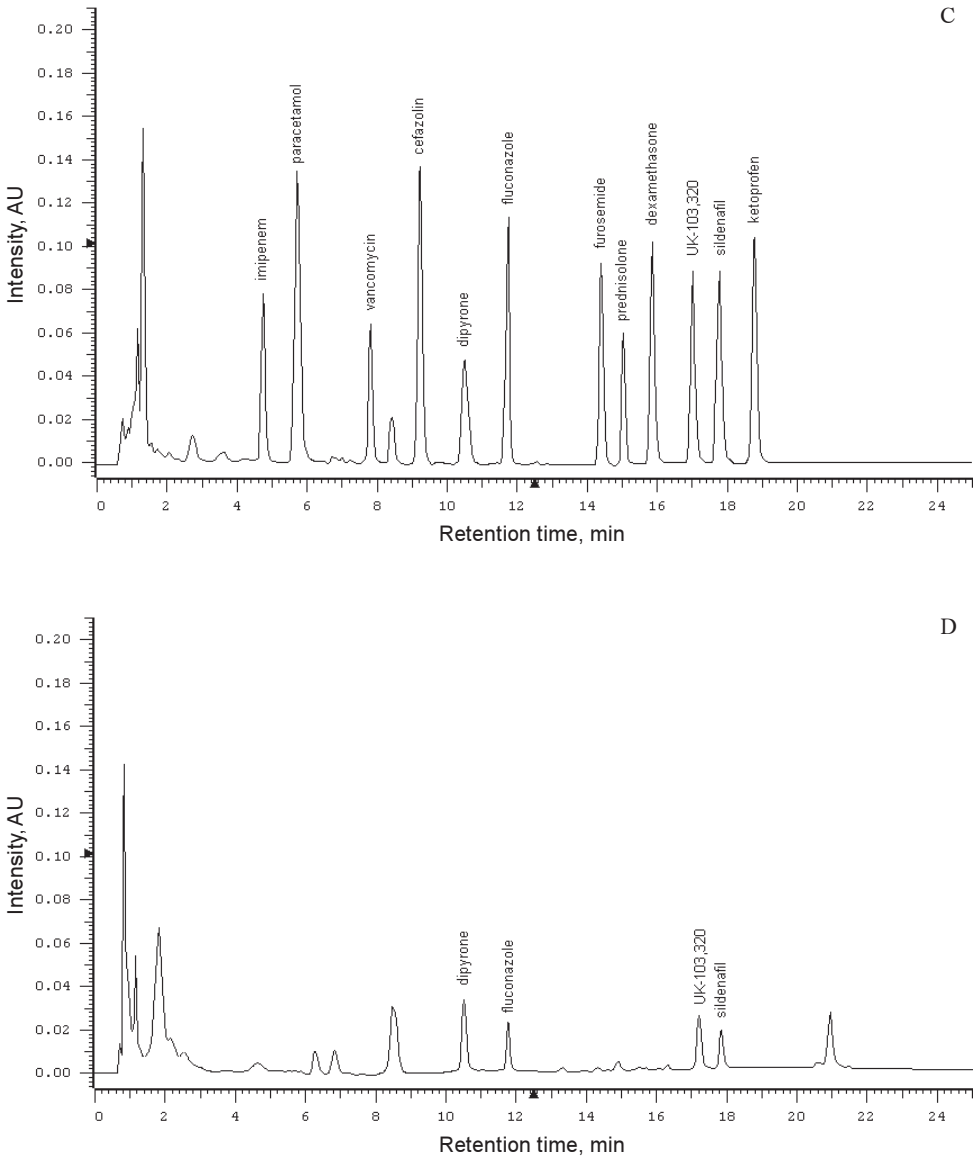

Figure 2. (Continuation)

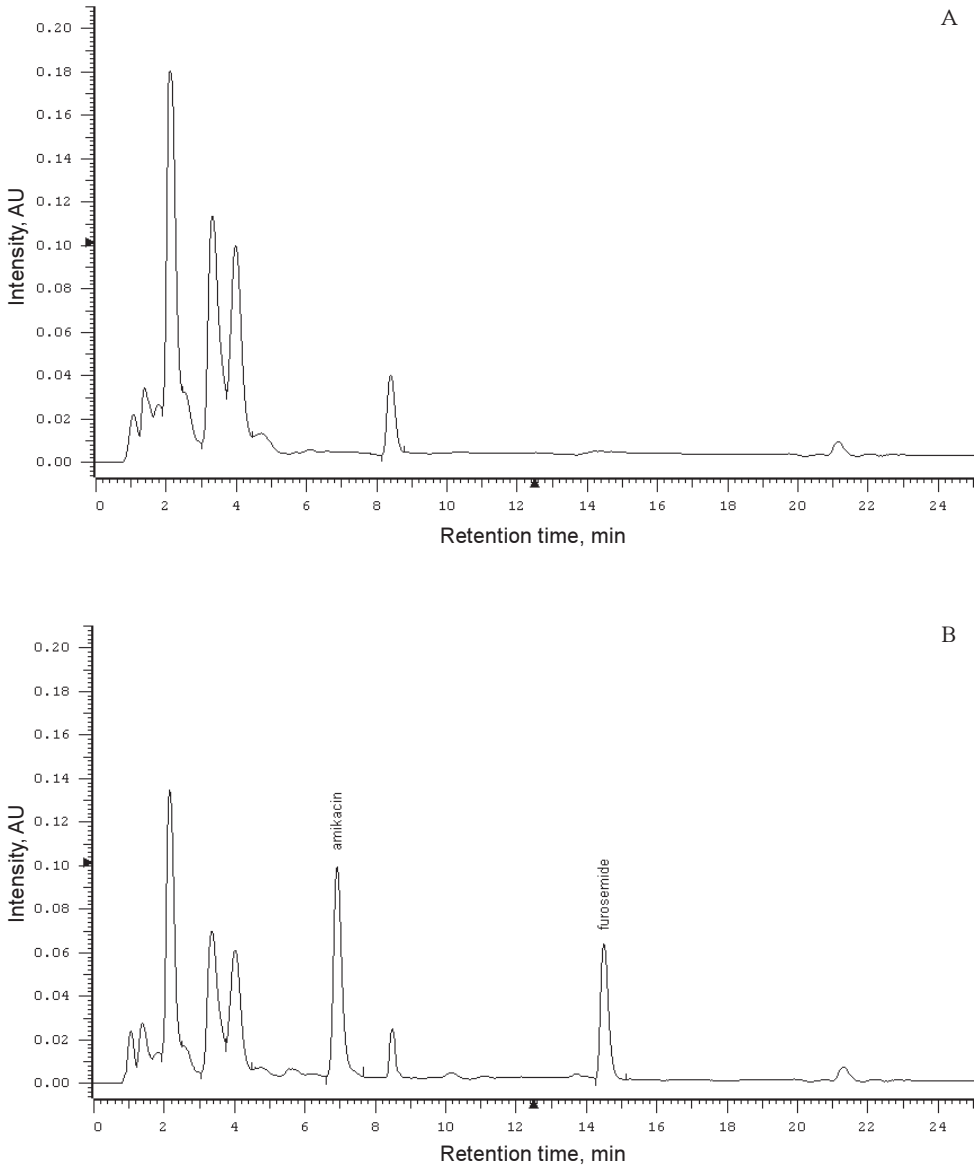

Figure 3. Representative typical RP–HPLC–FL chromatograms showing separation of the analytes after extraction and derivatization with OPA–3MPA reagent. Sample prepared from blank human urine (A) and a sample containing of amikacin and furosemide from spiked urine (B). Fluorescent wavelength: excitation 355 nm, emission 415 nm. Chromatographic conditions are described in text

The specificity of the method is enhanced by detecting the peaks at eleven different wavelengths. The maximum absorption of SC, UK–103,320, IMP, PAR, VCM, CFZ, DPR, FZ, FUR, PRE, DEX and KET were detected at 231, 231, 300, 243, 210, 274, 259, 210, 234, 242, 242 and 254 nm, respectively. These wavelengths were chosen for the analysis. Retention times of the compounds in this system are presented in Table 2.

Table 2. Retention times and C.V. for analysed drugs (n =10)

Analyte	Retention times of standards (mean ± S.D.[a]) min	C.V.[b] (%)	Retention times of standards in urine sample (mean ± S.D.[c]) min	C.V.[d] %
SC	17.68 ± 0.03	0.17	17.79 ± 0.04	0.22
UK–103,320	16.97 ± 0.03	0.18	17.04 ± 0.03	0.18
IMP	4.71 ± 0.02	0.42	4.76 ± 0.02	0.42
PAR	5.91 ± 0.02	0.34	5.84 ± 0.03	0.51
AMK	7.11 ± 0.02	0.28	7.02 ± 0.02	0.28
VCM	7.81 ± 0.03	0.38	7.87 ± 0.03	0.38
CFZ	9.40 ± 0.02	0.21	9.34 ± 0.02	0.20
DPR	10.59 ± 0.04	0.38	10.53 ± 0.03	0.28
FZ	11.83 ± 0.02	0.17	11.78 ± 0.02	0.17
FUR	14.17 ± 0.02	0.14	14.22 ± 0.03	0.21
PRE	15.12 ± 0.03	0.20	15.07 ± 0.03	0.19
DEX	16.02 ± 0.02	0.13	15.92 ± 0.02	0.12
KET	18.76 ± 0.03	0.16	18.84 ± 0.03	0.16

[a, c] Standard deviation of retention times (standard, in the human urine spiked, respectively).
[b, d] Coefficient of variation of retention times (standards, in the human urine spiked, respectively).

This system offers furthermore the opportunity for simultaneous determination of AMK and FUR in human urine samples (Fig. 3B). AMK was analysed after derivatization with OPA–3MPA using RP–HPLC with fluorescence detection. The OPA–3MPA reagent did not interfere with fluorescence detection of the AMK and FUR. In this system retention times for AMK and FUR were 7.02 and 14.22 min, respectively. The total run time of 25 min was sufficient for sample analysis that allows analysing large number of samples in a short period of time. The separation of investigated compounds showed a satisfactory precision, accuracy, linearity, recovery, specificity and sensitivity.

Drug identification was performed according to the standard addition method. Absorption spectra of the examined compounds were also compared.

Calibration curves and linearity

The linear regression analysis was carried out by plotting the peak areas „y" against the concentrations „x" of drugs. These concentrations used were based on the range expected during pharmacokinetic studies. The slopes, intercepts and correlation coefficients for the calibration curves of SC, UK–103,320 and other drugs are shown in Table 3. By examining the calibration curves and the table it can be noticed that the relationship between peak area ratio and concentration is linear within the studied concentration range. Calibration curve for FUR and its urine indication were done for RP–HPLC–DAD system because it provides determination of this drug in the presence of greatest amount of analysed drugs.

Linearity of the calibration curves for the thirteen drugs was achieved for concentrations between 0.02–50 μg mL^{-1}. Calibration curve for the fluorescent AMK derivative in human urine samples is linear in the range 0.50–32 μg mL^{-1}. The high correlation coefficients (r^2) of the all calibration curves (peak areas *vs* concentration) were between 0.9984 and 0.9996. The optimized method was validated by a standard procedure to evaluate if adequate precision, accuracy and linearity had been achieved. The prepared mixture of standards was injected 10 times as a test sample. From the respective area counts, the concentrations of the thirteen analysed drugs were calculated using the detector responses.

Precision and accuracy

The extraction procedure described earlier was carried out on urine samples spiked with the analytes. This chromatographic system was validated for both intra-day and inter-day precision.

The accuracy of the method was verified by the means of recovery studies, adding known amounts of SC, UK–103,320, IMP, PAR, AMK, VCM, CFZ, DPR, FZ, FUR, PRE, DEX and KET standard solutions to a known amount of human urine and subjecting the mixture to the usual extraction procedure (n = 10 for each concentration tested). The method proved to be very accurate (percent bias for all calibration samples varied from 0.04 to 8.00%) and precise (ranged from 0.38 to 6.98%). The assay method was validated with intra-day and inter-day variations less than 7%. The results for intra-day and inter-day precision (C.V.) and accuracy are summarized in Tables 4 and 5. The method meets all common requirements for accuracy, precision and linearity.

Table 3. Data on regression equations for determination of all examined analytes in human urine. S_{xy} residual standard deviation of regression coefficient; LOD limits of detection were established at a signal-to-noise ratio (S/N) of 3; LOQ limits of quantification were established at a signal-to-noise ratio (S/N) of 10

	Regression equation [a]									
Analyte	Linear dynamic range µg mL^{-1}	Slope [b] (mean ± S.D.)	C.V.[c] %	Intercept [b] (mean ± S.D.)	C.V.[d] %	Correlation coefficient [b] r^2 (n = 10) [f] (mean ± S.D.)	C.V.[e] %	S_{xy}	LOD µg mL^{-1}	LOQ µg mL^{-1}
SC	0.03–8.5	72511 ± 588	0.02	26245 ± 9995	0.43	0.9994 ± 0.0004	2.08	63004	0.01	0.03
UK–103,320	0.03–8.5	70552 ± 592	0.02	21567 ± 10058	0.33	0.9994 ± 0.0007	2.09	63399	0.01	0.03
IMP	0.40–40	33519 ± 159	0.03	–11568 ± 3349	0.70	0.9996 ± 0.0011	4.50	21510	0.13	0.39
PAR	0.45–45	137586 ± 587	0.02	947814 ± 12217	0.42	0.9991 ± 0.0005	2.76	80079	0.15	0.44
AMK	0.50–32	170529 ± 566	0.02	967724 ± 9610	0.32	0.9991 ± 0.0004	1.99	60577	0.17	0.48
VCM	0.20–25	25494 ± 70	0.03	–5153.6 ± 841	0.39	0.9990 ± 0.0002	2.41	5195	0.07	0.20
CFZ	0.02–50	192550 ± 583	0.02	271026 ± 13954	0.42	0.9993 ± 0.0001	2.81	93470	0.01	0.02
DPR	3.50–35	166587 ± 963	0.04	–368648 ± 19772	0.76	0.9993 ± 0.0009	3.32	86735	1.16	3.45
FZ	0.25–25	64209 ± 311	0.06	48846 ± 3420	0.63	0.9984 ± 0.0004	4.03	21901	0.08	0.25
FUR	0.02–20	36374 ± 1402	0.06	488441 ± 11623	0.48	0.9989 ± 0.0002	3.11	74994	0.01	0.02

(Continuation on the next page)

Table 3. (Continuation)

	Regression equation [a]									
Analyte	Linear dynamic range µg mL^{-1}	Slope [b] (mean ± S.D.)	C.V.[c] %	Intercept [b] (mean ± S.D.)	C.V.[d] %	Correlation coefficient [b] r^2 (n = 10) [f] (mean ± S.D.)	C.V.[e] %	S_{xy}	LOD µg mL^{-1}	LOQ µg mL^{-1}
PRE	0.10–22	115648 ± 331	0.03	58406 ± 3853	0.40	0.9985 ± 0.0001	2.61	25363	0.03	0.10
DEX	0.25–30	59574 ± 241	0.04	32766 ± 3550	0.53	0.9992 ± 0.0006	3.24	21748	0.07	0.25
KET	0.10–32	133428 ± 549	0.03	429334 ± 9969	0.45	0.9988 ± 0.0003	2.68	59712	0.03	0.09

[a] Calibration curves were fitted to the linear regression equation y = ax + b, where „y" represents the ratio of drug peak areas, „a", and „b" are constants, and „x" is the concentration of analysed drugs.

[b] Values are mean ± S.D. of calibration curves.

[c] Coefficient of variation of slope.

[d] Coefficient of variation of intercept.

[e] Coefficient of variation of regression coefficient.

[f] Number of points in calibration curves.

Table 4. Intra-day precision and accuracy of these analytes in human urine samples and absolute recoveries from spiked human urine (n = 10)

Analyte	Spiked concentration $\mu g\ mL^{-1}$	Calculated concentration $\mu g\ mL^{-1}$	S.D.[a] $\mu g\ mL^{-1}$	C.V.[b] %	Accuracy %	Absolute recovery %
SC	8.50	8.46	0.271	3.20	–0.53	95.12
	5.00	4.91	0.109	2.22	–1.78	94.85
	3.00	2.96	0.049	1.65	–1.20	96.21
	1.00	0.96	0.022	2.29	–3.80	95.33
	0.10	0.10	0.003	3.00	–3.00	94.70
UK–103,320	8.50	8.41	0.272	3.23	–1.05	98.13
	5.00	5.02	0.101	2.01	0.46	96.41
	3.00	2.95	0.074	2.51	–1.67	98.04
	1.00	1.02	0.019	1.86	2.30	98.25
	0.10	0.10	0.003	2.99	2.00	98.65
IMP	40.00	39.92	0.814	2.04	–0.19	59.01
	30.00	29.76	0.855	2.87	–0.82	59.51
	10.00	9.76	0.162	1.66	–2.37	62.13
	2.50	2.56	0.020	0.78	2.44	61.95
	0.50	0.53	0.003	0.56	6.40	59.48
PAR	45.00	44.30	0.802	1.81	–1.56	71.45
	25.00	24.83	0.509	2.05	–0.67	72.48
	10.00	10.03	0.249	2.48	0.29	73.56
	2.50	2.57	0.083	3.23	2.92	72.60
	1.00	0.98	0.063	6.40	–1.60	70.85
AMK	32.00	32.69	0.405	1.24	2.16	85.98
	20.00	19.95	0.370	1.85	–0.24	84.25
	10.00	10.08	0.205	2.03	0.83	86.02
	2.50	2.45	0.068	2.78	–1.96	87.05
	1.00	1.01	0.013	1.28	1.40	85.42

(Continuation on the next page)

Table 4. (Continuation)

Analyte	Spiked concentration µg mL^{-1}	Calculated concentration µg mL^{-1}	S.D.[a] µg mL^{-1}	C.V.[b] %	Accuracy %	Absolute recovery %
VCM	25.00	25.01	0.128	0.51	0.04	76.46
	15.00	15.25	0.203	1.33	1.69	77.40
	7.50	7.38	0.066	0.89	–1.55	74.60
	2.50	2.50	0.049	1.96	0.12	75.19
	0.50	0.51	0.007	1.36	2.60	74.90
CFZ	50.00	49.82	0.301	0.60	–0.36	83.25
	30.00	30.58	0.257	0.84	1.95	81.45
	10.00	9.87	0.248	2.51	–1.33	83.45
	1.00	0.99	0.024	2.42	–0.80	81.59
	0.10	0.10	0.002	1.90	5.00	82.02
DPR	35.00	35.34	0.922	2.61	0.96	71.41
	25.00	24.49	0.494	2.02	–2.03	71.90
	17.50	17.52	0.405	2.31	0.09	69.45
	12.50	12.75	0.149	1.17	2.03	71.23
	6.50	6.57	0.061	0.93	1.11	70.41
FZ	25.00	24.59	0.860	3.50	–1.63	64.25
	15.00	15.23	0.358	2.35	1.55	62.56
	5.00	5.04	0.176	3.49	0.80	63.07
	1.50	1.41	0.024	1.71	–6.27	
	0.50	0.47	0.012	2.54	–5.60	61.37
FUR	20.00	19.69	0.281	1.43	–1.55	89.45
	10.00	10.10	0.247	2.44	1.05	87.41
	5.00	4.94	0.282	5.71	–1.16	88.59
	1.00	0.98	0.037	3.76	–1.70	86.68
	0.10	0.10	0.003	3.12	–4.00	87.51

(Continuation on the next page)

Table 4. (Continuation)

Analyte	Spiked concentration µg mL^{-1}	Calculated concentration µg mL^{-1}	S.D.[a] µg mL^{-1}	C.V.[b] %	Accuracy %	Absolute recovery %
PRE	22.00	22.18	0.243	1.10	0.84	75.48
	15.00	14.91	0.156	1.04	–0.57	74.91
	5.00	4.96	0.019	0.38	–0.74	74.51
	1.00	0.95	0.011	1.16	–4.90	74.85
	0.25	0.24	0.001	0.41	–2.40	75.09
DEX	30.00	30.03	0.372	1.24	0.11	68.45
	20.00	20.21	0.884	4.37	1.03	67.01
	10.00	9.83	0.108	1.10	–1.73	68.40
	2.50	2.58	0.086	3.33	3.24	66.98
	0.50	0.52	0.010	1.90	5.00	68.14
KET	32.00	31.53	0.839	2.66	–1.46	89.52
	25.00	25.56	0.473	1.85	2.24	90.27
	12.50	12.75	0.588	4.61	1.98	91.25
	4.50	4.35	0.116	2.67	–3.42	91.02
	0.75	0.74	0.041	5.53	–1.07	90.62

[a] Standard deviation of concentrations found.

[b] Coefficient of variation of concentrations found.

Table 5. Inter-day precision and accuracy of these analytes in human urine samples and absolute recoveries from spiked human urine (n = 10)

Analyte	Spiked concentration $\mu g\ mL^{-1}$	Calculated concentration $\mu g\ mL^{-1}$	S.D.[a] $\mu g\ mL^{-1}$	C.V.[b] %	Accuracy %	Absolute recovery %
SC	6.00	6.15	0.214	3.48	2.50	96.15
	4.00	3.99	0.104	2.60	–0.22	95.08
	2.00	2.06	0.077	3.73	3.20	94.45
	0.50	0.50	0.021	4.17	0.60	96.09
	0.03	0.03	0.001	3.33	4.00	95.67
UK–103,320	6.00	6.17	0.191	3.09	2.87	97.85
	4.00	3.97	0.113	2.85	–0.75	97.12
	2.00	1.95	0.082	4.22	–2.75	96.90
	0.50	0.51	0.013	2.53	2.80	96.78
	0.03	0.03	0.001	3.34	8.00	97.42
IMP	35.00	35.25	1.580	4.48	0.72	61.42
	20.00	20.22	0.442	2.19	1.09	63.49
	5.00	5.01	0.119	2.37	0.26	60.26
	1.00	1.03	0.025	2.43	2.70	64.08
	0.40	0.38	0.002	0.52	–4.75	60.41
PAR	35.00	36.19	0.857	2.37	3.40	73.25
	15.00	14.60	0.497	3.40	–2.65	70.08
	5.00	5.05	0.157	3.11	1.08	71.05
	1.50	1.49	0.104	6.98	–0.73	70.54
	0.45	0.47	0.014	2.97	4.67	71.02
AMK	30.00	29.19	0.656	2.25	–2.70	87.45
	15.00	15.18	0.383	2.52	1.23	85.69
	5.00	4.95	0.126	2.54	–0.98	85.19
	1.50	1.47	0.043	2.92	–1.80	84.56
	0.50	0.52	0.023	4.39	4.80	84.25

(Continuation on the next page)

Table 5. (Continuation)

Analyte	Spiked concentration µg mL^{-1}	Calculated concentration µg mL^{-1}	S.D.[a] µg mL^{-1}	C.V.[b] %	Accuracy %	Absolute recovery %
VCM	20.00	19.91	0.460	2.31	–0.45	75.25
	10.00	9.80	0.201	2.05	–1.98	75.69
	5.00	5.14	0.139	2.71	2.70	76.07
	1.00	1.02	0.017	1.67	1.70	77.14
	0.20	0.21	0.003	1.42	6.00	75.24
CFZ	40.00	39.75	0.311	0.78	–0.62	81.69
	20.00	20.18	0.290	1.44	0.92	82.95
	5.00	4.80	0.227	4.73	–3.96	84.69
	0.50	0.51	0.009	1.75	2.60	80.98
	0.02	0.02	0.001	4.76	5.00	81.65
DPR	30.00	30.18	0.494	1.64	0.61	70.56
	20.00	19.71	0.822	4.17	–1.43	72.10
	15.00	14.92	0.356	2.39	–0.56	70.01
	10.00	9.85	0.193	1.96	–1.50	72.56
	3.50	3.70	0.049	1.32	5.74	70.45
FZ	18.50	18.85	0.487	2.58	1.91	63.01
	10.00	10.07	0.180	1.79	0.67	64.45
	2.50	2.43	0.056	2.31	–2.96	62.08
	1.00	0.95	0.032	3.36	–4.80	63.15
	0.25	0.24	0.005	2.06	–2.80	62.43
FUR	12.50	13.01	0.196	1.51	4.08	88.25
	7.50	7.43	0.224	3.02	–0.95	89.05
	2.50	2.37	0.041	1.73	–5.04	87.40
	0.25	0.24	0.004	1.65	–2.80	88.19
	0.02	0.02	0.001	4.98	–8.00	86.92

(Continuation on the next page)

Table 5. (Continuation)

Analyte	Spiked concentration µg mL^{-1}	Calculated concentration µg mL^{-1}	S.D.[a] µg mL^{-1}	C.V.[b] %	Accuracy %	Absolute recovery %
PRE	20.00	19.79	0.345	1.74	–1.06	76.12
	10.00	10.19	0.196	1.92	1.95	75.27
	2.50	2.55	0.034	1.34	1.84	73.69
	0.50	0.50	0.012	2.39	0.40	76.98
	0.10	0.10	0.001	1.03	–3.00	74.11
DEX	25.00	25.06	0.344	1.37	0.24	69.45
	15.00	14.67	0.477	3.25	–2.21	67.51
	5.00	5.09	0.109	2.14	1.88	69.45
	1.00	1.04	0.012	1.15	4.00	69.36
	0.25	0.26	0.007	2.68	4.40	67.04
KET	30.00	29.92	0.597	2.00	–0.27	91.09
	17.50	17.67	0.232	1.31	0.99	91.46
	8.00	7.82	0.116	1.48	–2.19	89.76
	1.50	1.44	0.062	4.30	–4.00	89.45
	0.10	0.09	0.006	6.38	–6.00	90.08

[a] Standard deviation of concentrations found.
[b] Coefficient of variation of concentrations found.

Analytes recoveries

The one-step LLE has been successfully applied to the extraction of analysed drugs from human urine. The extraction recoveries of examined compounds from human urine samples were determined at different concentrations. These samples were subjected to the LLE procedure described earlier and injected into the RP–HPLC system (Figs. 2C and 3B). The highest recoveries were obtained for SC (from 94.45 to 96.21%), UK–103,320 (from 96.41 to 98.65%) and KET (from 89.45 to 91.46%). The lowest recoveries were obtained for IMP (from 59.01 to 64.08%), FZ (61.37 to 64.52%) and DEX (from 66.98 to 69.45%).

Furthermore, the basic extraction step reduced the quantities of endogenous substances no extracted with mixture of ethyl acetate–dichloromethane–chloroform (45:35:20, v/v/v) from human urine. A summary of the absolute recovery results is given in Tables 4 and 5. A percentage of recovery shows that the method can be successfully used on

a routine basis. The data proved the suitability of mixture for the extraction of investigated compounds from human urine samples.

Specificity

To evaluate the specificity of the method, 3 mL of drug-free urine was carried through the assay procedure and the retention times of endogenous compounds in urine were compared with those of SC and UK–103,320 (Fig. 1). No interfering peaks were observed near the retention time of analysed drugs in ten batches of human urine samples. There are some additional unidentified peaks in the chromatograms from the human urine samples, but these peaks do not interfere with the drugs of interest. Specificity of the method was assessed to test matrix influence between different urine samples (n = 10) from hospitalized patients.

Limits of detection and quantification

The limits of detection (LOD) and limits of quantification (LOQ) were estimated in accordance with the baseline noise method. The LOD were established at a signal-to--noise ratio (S/N) of 3. The LOQ were established at a signal-to-noise ratio (S/N) of 10. LOD and LOQ were experimentally appointed by ten injections of mixtures (SC, UK–103,320, IMP, PAR, AMK, VCM, CFZ, DPR, FZ, FUR, PRE, DEX and KET) at the LOD and LOQ concentrations. The noise of measurements was based on ten self--contained blank urine samples (urine without any added analytes, previously prepared according to all procedure described in paragraph Samples preparation). The respective values for the thirteen analytes are reported in Table 3.

Application of the method to patient urine samples

Human urine samples obtained from patients treated with SC, suffering from pulmonary hypertension have been tested. The separation from other drugs that could be co-administered in an intensive care unit was also studied. These urine samples were extracted and analysed as described above. This method can be successfully applied to routine simultaneous determination of SC, UK–103,320 and remaining examined drugs in human urine samples.

In analysed samples, one or more drugs from the thirteen examined compounds were detected. The concentrations of examined drugs in urine samples ranged between: 0.15–1.25 of SC, 0.10–2.2 of UK–103,320, 0.8–10.5 of PAR, 4.2–13.5 of DPR, 0.05–7.5 of CFZ and 1.3–11.5 μg mL^{-1} of FUR. Precision of signs for urine samples coming from patients was the same as for urine samples after addition of the examined drugs. The chromatogram of extracted urine sample obtained from patient treated with SC,

suffering from pulmonary hypertension is shown in Figure 2D. In urine samples coming from patients, no interference of the matrix was observed.

CONCLUSION

Application of RP–HPLC method for accurate measurements of SC, its metabolite UK–103,320 and eleven other drug in human urine from patients suffering from pulmonary hypertension was elaborated. In this study we have developed a simple and reliable procedure for the determination of SC, UK–103,320, IMP, PAR, AMK, VCM, CFZ, DPR, FZ, FUR, PRE, DEX and KET in human urine by gradient RP–HPLC with ultraviolet or fluorescence detection.

RP–HPLC procedure, using DAD and FL detectors, with very good precision, accuracy, linearity and recovery has been developed and validated for simultaneous separation and determination of SC, UK–103,320 and remaining analysed drugs in human urine. The very good sensitivity and selectivity make this method a valuable tool in clinical and basic research on the metabolism of SC and their role in the treatment of pulmonary hypertension.

Simple, fast and effective sample preparation for analysis, as well as short analysis time makes the elaborated procedure useful and possibile to apply in medical laboratory. To the best of our knowledge, the RP–HPLC method described herein is the first procedure allowing simultaneous detection and quantification of twelve drugs and one active metabolite.

Practical implications of our work rely on the possibility of simultaneous separation and analysis of several drugs in the same chromatography system. The good validation criteria results of the method allowed its use in the quantification of these compounds.

In conclusion, the HPLC assay method, developed in this study using a reversed--phase system was proved to be acceptable for assaying drugs in human urine samples, for patients treated for pulmonary hypertension.

Acknowledgements

This work was supported from science funds for scientific research for years 2007-2009, as Project No. N20402532/0738.

The authors would like to warmly thank Pfizer Global Research & Development for received standards SC and UK–103,320.

REFERENCES

1. Lepore J.J., Maroo A., Pereira N.L., Ginns L.C., Dec G.W., Zapol W.M., Bloch K.D. and Semigran M.J., *Amer. J. Card.*, **90**, 677 (2002).
2. Dinesh N.D., Vishukumar B.K., Nagaraja P., Made Gowda N.M. and Rangappa K.S., *J. Pharm. Biomed. Anal.*, **29**, 743 (2002).
3. Kim J., Ji H.Y., Kim S.J., Lee H.W., Lee S.S., Kim D.S., Yoo M., Kim W.B. and Lee H.S., *J. Pharm. Biomed. Anal.*, **32**, 317 (2003).
4. Rodríguez J., Berzas J.J., Castaneda G. and Rodríguez N., *Talanta*, **62**, 427 (2004).
5. Liaw J. and Chang T.W., *J. Chromatogr. B*, **765**, 161 (2001).
6. Cooper J.D.H., Muirhead D.C., Taylor J.E. and Baker P.R., *J. Chromatogr. B*, **701**, 87 (1997).
7. Guermouche M.H. and Bensalah K., *J. Pharm. Biomed. Anal.*, **40**, 952 (2006).
8. Saisho K., Scott K.S., Morimoto S. and Nakahara Y., *Biol. Pharm. Bull.*, **24**, 1384 (2001).
9. Nevado J.J.B., Flores J.R., Penalvo G.C. and Farinas N.R., *J. Chromatogr. A*, **953**, 279 (2002).
10. Eerkes A., Addison T. and Naidong W., *J. Chromatogr. B*, **768**, 277 (2002).
11. Jeong C.K., Lee H.Y., Jang M.S., Kim W.B. and Lee H.S., *J. Chromatogr. B*, **752**, 141 (2001).
12. Podlewski J.K., Chwalibowska-Podlewska A., *Leki współczesnej terapii*, Wyd. XVIII, Split Trading, Warszawa 2007, (in Polish).
13. Garcia-Capdevila L., López-Calull C., Arroyo C., Moral M.A., Mangues M.A. and Bonal J., *J. Chromatogr. B*, **692**, 127 (1997).
14. Monser L. and Darghouth F., *J. Pharm. Biomed. Anal.*, **27**, 851 (2002).
15. Yang M. and Tomellini S.A., *J. Chromatogr. A*, **939**, 59 (2001).
16. Ghassempour A., Darbandi M. K. and Asghari F.S., *Talanta*, **55**, 573 (2001).
17. Adamczyk M., Brate E.M., Chiappetta E.G., Ginsburg S., Hoffman E., Klein C., Perkowitz M.M., Rege S.D., Chou P.P. and Costantino A.G., *Ther. Drug Monit.*, **20**, 191 (1998) .
18. Matzke G.R., Zhanel G.G. and Guay D.R.P., *Clin. Pharmacokin.*, **11**, 257 (1986).
19. Al-Rawithi S., Hussein R., Raines D.A., Al-Showaier I. and Kurdi W., *J. Pharm. Biomed. Anal.*, **22**, 281 (2000).
20. Altun M.L., *Turk. J. Chem.*, **26**, 521 (2002).
21. Cociglio M., Brandissou S., Alric R. and Bressolle F., *J. Chromatogr. B*, **686**, 11 (1996).
22. Nava-Ocampo A.A., Velázquez-Armenta E.Y., Reyes-Pérez H., Ramirez-Lopez E. and Ponce-Monter H., *J. Chromatogr. B*, **730**, 49 (1999).
23. Döppenschmitt S.A., Scheidel B., Harrison F. and Surmann J.P., *J. Chromatogr. B*, **674**, 237 (1995).
24. Milojevic Z., Agbaba D., Eric S., Boberic-Borojevic D., Ristic P. and Solujic M., *J. Chromatogr. A*, **949**, 79 (2002).
25. Jalón E.G., Josa M., Campanero M.A., Santoyo S. and Ygartua P., *J. Chromatogr. A*, **870**, 143 (2000).
26. Carlucci G., Biordi L., Vicentini C. and Bologna M., *J. Pharm. Biomed. Anal.*, **8**, 283 (1990).
27. Bloh A.M., Campos J.M., Alpert G. and Plotkin S.A., *J. Chromatogr.*, **375**, 444 (1986).
28. Myers C.M. and Blummer J.L., *Antimicrob. Agents Chemother.*, **26**, 78 (1984).
29. Gravallese D.A., Musson D.G., Pauliukonis L.T. and Bayne W.F., *J. Chromatogr.*, **310**, 71 (1984).
30. Di Girolamo A., O'Neill W.M. and Wainer I.W., *J. Pharm. Biomed. Anal.*, **17**, 1191 (1998).
31. Nagaralli B.S., Seetharamappa J., Gowda B.G. and Melwanki M.B., *J. Chromatogr. B*, **798**, 49 (2003).
32. Pufal E., Sykutera M., Rochholz G., Schütz H.W., Sliwka K. and Kaatsch H.-J., *Fresen. J. Anal. Chem.*, **367**, 596 (2000).

33. Vertzoni M.V., Archontaki H.A. and Galanopoulou P., *J. Pharm. Biomed. Anal.*, **34**, 487 (2003).
34. Kamberi M., Riley C.M., Ma (Sharon) X. and Huang C.-W.C., *J. Pharm. Biomed. Anal.*, **34**, 123 (2004).
35. Jensen L.S., Valentine J., Milne R.W. and Evans A.M., *J. Pharm. Biomed. Anal.*, **34**, 585 (2004).
36. Oliveira E.J., Watson D.G. and Morton N.S., *J. Pharm. Biomed. Anal.*, **29**, 803 (2002).
37. Campanero M.A., Calahorra B., García-Quetglas E., López-Ocáriz A. and Honorato J., *J. Pharm. Biomed. Anal.*, **20**, 327 (1999).
38. Shervington L.A. and Sakhnini N., *J. Pharm. Biomed. Anal.*, **24**, 43 (2000).
39. Morovjan G., Csokan P.P. and Németh-Konda L., *Chromatographia*, **48**, 32 (1998).
40. Lung K.R., Kassal K.R., Green J.S. and Hovsepian P.K., *J. Pharm. Biomed. Anal.*, **16**, 905 (1998).
41. Adams E., Vaerenbergh G., Roets E. and Hoogmartens J., *J. Chromatogr. A*, **819**, 93 (1998).
42. Hosotsubo H., *J. Chromatogr.*, **487**, 421 (1989).
43. Furuta I., Kitahashi T., Kuroda T., Nishio H., Oka C. and Morishima Y., *Clin. Chim. Acta*, **301**, 31 (2000).
44. Lukša J. and Marušič A., *J. Chromatogr. B*, **667**, 277 (1995).
45. Del Nosal M.J., Bernal J.L., Pampliega A., Marinero P., López M.I. and Coco R., *J. Chromatogr. A*, **727**, 231 (1996).
46. Farin D., Piva G.A., Gozlan I. and Kitzes-Cohen R., *J. Pharm. Biomed. Anal.*, **18**, 367 (1998).
47. Favetta P., Guitton J., Bleyzac N., Dufresne C. and Bureau J., *J. Chromatogr. B*, **751**, 377 (2001).
48. Backes D.W., Aboleneen H.I. and Simpson J.A., *J. Pharm. Biomed. Anal.*, **16**, 1281 (1998).
49. Najjar T.A., Al-Dhuwailie A.A. and Tekle A., *J. Chromatogr. B*, **672**, 295 (1995).
50. Metz P., Kohlhepp S.J. and Gilbert D.N., *J. Chromatogr. B*, **773**, 159 (2002).
51. Senyuva H.Z., Aksahin I., Ozcan S. and Kabasakal B.V., *Anal. Chim. Acta*, **547**, 73 (2005).
52. Mathy F.X., Vroman B., Ntivunwa D., De Winne A.J. Verbeeck R.K. and Préat V., *J. Chromatogr. B*, **787**, 323 (2003).
53. Lee C.H., Yeh P.H. and Tsai T.H., *Inter. J. Pharm.*, **241**, 367 (2002).
54. Abdel-Moety E.M., Khattab F.I., Kelani K.M. and AbouAl-Alamein A.M., *Il Farmaco*, **57**, 931 (2002).
55. Koks C.H.W., Rosing H., Meenhorst P.L., Bult A. and Beijnen J.H., *J. Chromatogr. B*, **663**, 345 (1995).
56. Uchino K., Isozaki S., Saitoh Y., Nakagawa F. and Tamura Z., *J. Chromatogr. Biomed. Appl.*, **308**, 241 (1984).
57. Abou-Auda H.S., Al-Yamani M.J., Morad A.M., Bawazir S.A., Khan S.Z. and Al-Khamis K.I., *J. Chromatogr. B*, **710**, 121 (1998).
58. Abdel-Hamid M.E., *Il Farmaco*, **55**, 448 (2000).
59. Okuda T., Yamashita K. and Motohashi M., *J. Chromatogr. B*, **682**, 343 (1996).
60. Mills C.D., Whitworth C., Rybak L.P. and Henley C.M., *J. Chromatogr. B*, **701**, 65 (1997).
61. El-Saharty Y.S., *J. Pharm. Biomed. Anal.*, **33**, 699 (2003).
62. Cirimele V., Kintz P., Dumestre V., Goullé J. and Ludes B., *Forensic. Sci. Int.*, **107**, 381 (2000).
63. AbuRuz S., Millership J., Heaney L. and McElnay J., *J. Chromatogr. B*, **798**, 193 (2003).
64. Tobita T., Senarita M., Hara A. and Kusakari J., *Hearing Research*, **165**, 30 (2002).
65. Volin P., *J. Chromatogr. B*, **671**, 319 (1995).
66. Nozaki O., *J. Chromatogr. A*, **935**, 267 (2001).
67. Shibasaki H., Furuta T. and Kasuya Y., *J. Chromatogr. B*, **692**, 7 (1997).
68. Frerichs V.A. and Tornatore K.M., *J. Chromatogr. B*, **802**, 329 (2004).
69. Neufeld E., Chayen R. and Stern N., *J. Chromatogr. B*, **718**, 273 (1998).

70. Shibata N., Hayakawa T., Takada K., Hoshino N., Minouchi T. and Yamaji A., *J. Chromatogr. B*, **706**, 191 (1998).
71. Volin P., *J. Chromatogr. B*, **666**, 347 (1995).
72. Hashem H. and Jira Th., *Chromatographia*, **61**, 133 (2005).
73. Hay M. and Mormede P., *J. Chromatogr. B*, **702**, 33 (1997).
74. Zurbonsen K., Bressolle F., Solassol I., Aragon P.J., Culine S. and Pinguet F., *J. Chromatogr. B*, **804**, 421 (2004).
75. Grippa E., Santini L., Castellano G., Gatto M.T., Leone M.G. and Saso L., *J. Chromatogr. B*, **738**, 17 (2000).
76. Lemus Galleo J.M. and Pérez Arroyo J., *J. Pharm. Biomed. Anal.*, **30**, 1255 (2002).
77. Ameyibor E. and Stewart J.T., *J. Pharm. Biomed. Anal.*, **17**, 83 (1998).
78. Carr R.A., Caillé G., Ngoc A.H. and Foster R.T., *J. Chromatogr. B*, **668**, 175 (1995).
79. Lovlin R., Vakily M. and Jamali F., *J. Chromatogr. B*, **679**, 196 (1996).
80. Zakeri-Milani P., Barzegar-Jalali M., Tajerzadeh H., Azarmi Y. and Valizadeh H., *J. Pharm. Biomed. Anal.*, **39**, 624 (2005).
81. Martín M.J., Pablos F. and González A.G., *Talanta*, **49**, 453 (1999).
82. Molero-Monfort M., Escuder-Gilabert L., Villanueva-Camanas R.M., Sagrado S. and Medina-Hernandez M.J., *J. Chromatogr. B*, **753**, 225 (2001).
83. Vial J., Ménier I., Jardy A., Amger P., Brun A. and Burbaud L., *J. Chromatogr. B*, **708**, 131 (1998).
84. Dvořák J., Hájková R., Matysová L., Nováková L., Koupparis M.A. and Solich P., *J. Pharm. Biomed. Anal.*, **36**, 625 (2004).
85. Zhang W.-Z. and Kaye D.M., *Anal. Biochem.*, **326**, 87 (2004).
86. Baranowska I., Markowski P. and Baranowski J., *Anal. Chim. Acta*, **570**, 46 (2006).

Received February 2007
Revised July 2007
Accepted July 2007