Talanta 81 (2010) 871–880

Contents lists available at ScienceDirect

Talanta

journal homepage: www.elsevier.com/locate/talanta

Simultaneous determination of 11 antibiotics and their main metabolites from four different groups by reversed-phase high-performance liquid chromatography–diode array–fluorescence (HPLC–DAD–FLD) in human urine samples

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ARTICLE INFO

Article history:
Received 15 October 2009
Received in revised form 11 January 2010
Accepted 17 January 2010
Available online 25 January 2010

Keywords:
Drugs
Antibiotics
Human urine
SPE
HPLC

ABSTRACT

A new, accurate and sensitive reversed-phase high-performance liquid chromatography (RP-HPLC) as analytical method for the quantitative determination of 11 antibiotics (drugs) and the main metabolites of five of them present in human urine has been worked out, optimized and validated. The analytes belong to four different groups of antibiotics (sulfonamides, tetracyclines, penicillins and anphenicols). The analyzed compounds were sulfadiazine (SDI) and its N^4-acetylsulfadiazine (NDI) metabolite, sulfamethazine (SMZ) and its N^4-acetylsulfamethazine (NMZ), sulfamerazine (SMR) and its N^4-acetylsulfamerazine (NMR), sulfamethoxazole (SMX), trimetroprim (TMP), amoxicillin (AMX) and its main metabolite amoxicilloic acid (AMA), ampicillin (AMP) and its main metabolite ampicilloic acid (APA), chloramphenicol (CLF), thiamphenicol (TIF), oxytetracycline (OXT) and chlortetracycline (CLT). For HPLC analysis, diode array (DAD) and fluorescence (FLD) detectors were used. The separation of the analyzed compounds was conducted by means of a Phenomenex® Gemini C_{18} (150 mm × 4.6 mm I.D., particle size 5 μm) analytical column with LiChroCART® LiChrospher® C_{18} (4 mm × 4 mm, particle size 5 μm) guard column. Analyzed drugs were determined within 34 min using formic acid 0.1% in water and acetonitrile in gradient elution mode as mobile phase. A linear response was observed for all compounds in the range of concentration studied. Two procedures were optimized for sample preparation: a direct treatment with methanol and acetonitrile and a solid phase extraction procedure using Bond Elut® Plexa™ columns. The method was applied to the determination of the analytes in human urine from volunteers under treatment with different pharmaceutical formulations. This method can be successfully applied to routine determination of all these drugs in human urine samples.

1. Introduction

Antimicrobials are widely prescribed with both therapeutical and prophylactic purposes against microbial infections. It often happens that patients are treated simultaneously with antibiotics from different groups. Therefore it is necessary to develop chromatographic procedures which could determine various drugs simultaneously in the shortest time.

In this study, the selection of drugs was made according to the most often prescribed antibiotics for common infections and combinations of these frequently used. Combinations of drugs can be also found in urine samples from patients whose postoperative course was complicated by infection, sepsis and multi organ dysfunction or failure syndrome. As a result of this, hospitals are more often demanding fast and simple methods that permit the determination of a wide range of drugs belonging to different groups or therapeutic classes in just one analysis for both screening or quantitative purposes to dosing adjustment or for patients with unknown treatments to avoid combinations of drugs which are not recommended to be used simultaneously.

Liquid chromatography–mass spectrometry (LC–MS) and especially LC–MS-MS applied in the pharmaceutical industry, is a powerful tool for drug determination. LC–MS analyses are now achieved in less than 10 min [1–3] while 1.5–2 min separation times are increasingly common [4–6], but the use of these methodology requires costly instrumentation and qualified personal which is not always available in routine analysis of medical laboratories, and the application to a complex matrix as urine do not always give good results. The need of methods implying more simple and less sophisticated methodology is a fact.

Sulfadiazine (SDI), sulfamethazine (SMZ), sulfamerazine (SMR), sulfamethoxazol (SMX) and trimetroprim (TMP) are sulfonamides

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0039-9140/$ – see front matter © 2010 Elsevier B.V. All rights reserved.
doi:10.1016/j.talanta.2010.01.031

which are a group of antibacterial agents commonly used in urinary tract infectious, pneumocystis pneumonia, chronic bronchitis, meningococcal meningitis, acute otitis media or toxoplasmosis. Several methods have been reported for the detection of sulfonamides alone or in combination with other sulfonamides including photometric method [7], the Bratton–Marshall method [8,9], titrimetric assay method [10], high-performance liquid chromatography [11–15], gas chromatography and gas chromatography–mass spectrometry [16,17] and capillary electrophoresis (CE) [18,19].

Amoxicillin (AMX) and ampicillin (AMP) are penicillins which have been the most widely used antimicrobial drugs for more than 80 years and they are still considered as one of the most important groups of antibiotics. They are broad-spectrum antibiotics, clinically used against Gram-positive and Gram-negative bacteria. Amoxicillin is an α-amino-substituted β-lactam antibiotic frequently used because of its broad spectrum and low cost [20]. AMX has a bactericidal action and inhibits the bacterial cell-wall biosynthesis by binding to the enzymes which generate the protein cell wall [21]. The two major metabolites of amoxicillin are amoxicilloic acid (AMA) and amoxicillin diketopiperazine-2′,5′-dione. These metabolites have lost the antibacterial activity of the parent component [22], and AMA could have potential allergic properties [23]. Several analytical methods have been developed for the determination of penicillin antibiotics. Most of them involve HPLC and are applied to the analysis of AMX [24–28]. Methods for the simultaneous analysis of a range of penicillins have been also developed [25,26,29]. Direct UV detection is mainly used at low wavelengths [24–28] and fluorimetric detection is also applied after pre-column or post-column derivatization to improve sensitivity [20,30]. Other techniques for AMX analysis in pharmaceuticals involve CE [20] and FIA [31].

Ampicillin, as well as amoxycillin, is widely used β-lactam antibiotics belonging to the penicillin group. Ampicillin is very effective against Gram-negative and Gram-positive bacteria. It is used in treating infections of the intestinal, urinary and respiratory tracts. Ampicillin shows lower antibacterial activity than amoxycillin. It has worse antibacterial effect against the active streptococus hemolyticus, pneumococcus, diphtheria as well as bacillocon. High-performance liquid chromatographic (HPLC) methods have been developed previously for the determination of ampicillin in biological fluids [32–38].

Oxytetracycline (OXT) and chlortetracycline (CLT) belong to tetracycline class. They are important tetracyclines characterized by a broad-spectrum activity against pathogenic microorganisms [39]. Spectrophotometric methods are common [40,41] but the most widely used technique is reversed-phase HPLC [42,43]. There are examples of the determination of OXT and its degradation products in synthetic and biological samples by capillary zone electrophoresis (CZE) [44] and micellar electrokinetic chromatography (MEKC) [45].

Chloramphenicol (CLF) is a broad-spectrum antibiotic used in both, human and veterinary medicine. It is a potent drug for the treatment of childhood meningitis and typhoid fever [46], but toxic effects such as bone-marrow suppression, aplastic anemia and the 'Grey Baby Syndrome', have restricted its use. Thus, the monitoring of chloramphenicol levels in patients and its detection in food products are highly important. Currently used methods for the detection of chloramphenicol include chromatographic [47,48], microbiological [49], enzymatic [50], immunological [51] and other assays which are often laborious and expensive, hence simpler and more rapid methods are required.

Thiamphenicol is an analogue of chloramphenicol in which the nitro group on the benzene ring is replaced with a methylsulfonic group. It was reported that TMP shows particular therapeutic effect in respiratory infections, bacterial prostatitis and venereal diseases [52]. Some papers have reported determination methods for TMP in biological samples [53–56].

The aim of this work is the development of a new chromatographic method and the optimization of all parameters for the simultaneous determination of AMA, AMX, SDI, APA, TMP, AMP, SMR, OXT, SMZ, TIF, CLT, SMX, NDI, CLF, NMR and NMZ. Table 1 shows their structures and IUPAC names. Two extraction methods have been optimized and compared and a chromatographic method has been developed, validated and applied to the determination of all drugs in human urine samples. Simple and effective sample preparation for analyses as well as relatively short time of these analyses prove its usefulness and applicability in clinical laboratories.

2. Material and methods

2.1. Chemical and reagents

AMX, AMP, SDI, SMR, SMZ, SMX, TMP, CLF, TIF, OXT and CLT (97–99.9% purity) were purchased from Sigma–Aldrich Quimica, S.A. (Madrid, Spain). NMR, NMZ, NDI, AMA acid and APA were synthesized in our laboratory according to the protocols described by Baker [57] and Pfeifer et al. [58] and purity was checked by thin layer chromatography and mass spectrometry. Methanol, acetonitrile and dichloromethane for analysis (HPLC grade), were obtained from Romil Ltd. (Waterbeach, Cambridge, UK). Formic acid (98–100% purity) and sodium hydroxide, all analytical grade, were purchased from VWR (Barcelona, Spain). Phosphoric acid (85% purity) and ammoniac (30% purity) analytical grade were purchased from Panreac (Castellar del Vallés, Spain). Deionized water was obtained from a Milli-Q plus water system Millipore (Billerica, MA, USA).

Individual standard solutions of AMX, AMP, OXT, TIF, CLT, CLF, AMA and APA at a concentration of 500 μg mL^{-1} and 50 μg mL^{-1} of SDI, SMR, SMZ and SMX were prepared by dissolving the drugs in deionized water. Individual standard 500 μg mL^{-1} solutions of TMP, NDI, NMR and NMZ were prepared by dissolving the drugs in methanol. All standard solutions were stored at 4 °C and AMX, AMP, TIF, CLF, SDI, SMR, SMZ, SMX, TMP, NDI, NMR and NMZ were stable for at least one month; AMA and APA were daily prepared before use and were stable for 24 h; OXT and CLT stock solutions were stable for one week.

For validation of the method, human urine was obtained from pooled samples collected from healthy volunteers and stored at −18 °C before use.

2.2. Instrumentation

All measurements were made with an Agilent (Palo Alto, CA, USA) 1100 series liquid chromatograph equipped with a diode array and fluorescence detector connected in series mode, an injector with a loop of 20 μL, a quaternary pump, a vacuum degasser and a thermostated column compartment. Separations were carried out using a reversed-phase Phenomenex® Gemini C_{18e} 110 Å analytical column (150 mm × 4.6 mm, particle size 5 μm) preceded by a guard-column LiChroCART® 4-4 LiChrospher® 100 RP-18 (4 mm × 4 mm, particle size 5 μm) (Merck, Darmstadt, Germany). A Sigma centrifuge Laborzentrifugen 4–10 (Osterode, Germany) was used to centrifuge human urine samples. Data acquisition was performed by a HPLC System Agilent-Technologies 1100.

2.3. Chromatographic separation

Chromatographic separations were performed at 30 °C. The mobile phase consisted of a mixture of 0.1% formic acid in water (component A) and acetonitrile (component B). A gradient elution

program at a 0.7 mL min^{-1} flow rate was used to achieve the separation. The program begins with an isocratic step at 99% A for 7 min followed by a linear elution gradient from 99% to 70% A in 28 min more, and returned to initial conditions. Five minutes were waited between injections. The column effluent was monitored by DAD detector in the range of 200–450 nm, and FLD detection. All drugs (except TIF) and their main metabolites were detected using DAD. TIF was detected using only the fluorescence signal and AMA, AMX, SMR, SMX, NDI, NMR and NMZ were detected using both detectors. Table 2 shows the monitoring wavelengths for all the analyzed drugs and their retention times. To confirm the identification of the substances, the UV and the fluorescence spectra of the chromatographic peaks in urine samples and the standard solution were compared.

2.4. Sample preparation and collection

2.4.1. Procedure 1

500 μL of urine samples, pH adjusted to 8.0 with NaOH 1.5 M, were transferred to 5 mL tubes, 1.5 mL of methanol and 1.5 mL of acetonitrile were added, the tubes were mixed well and the suspension obtained were centrifuged for 5 min at 5000 × *g*. The

Table 1
Structures and IUPAC name of the examined antibiotics.

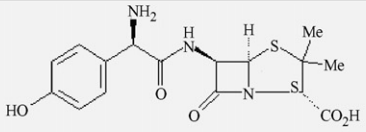
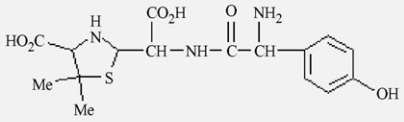
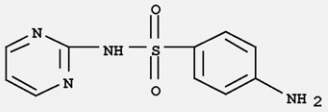
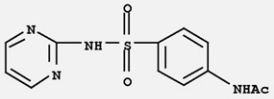
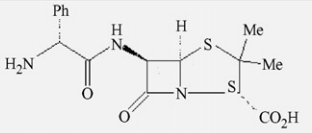
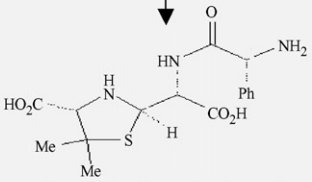
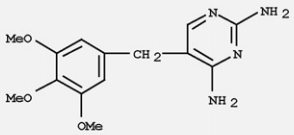
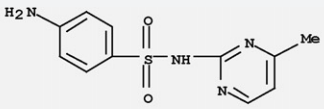
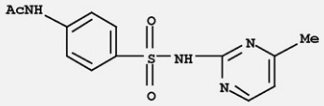
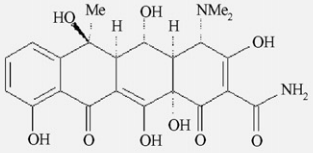
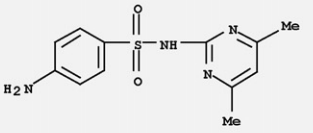
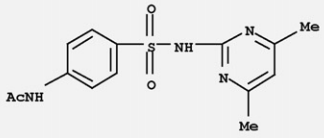
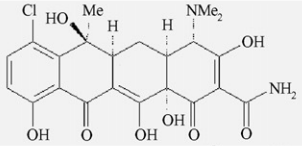
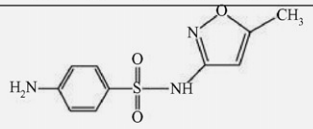
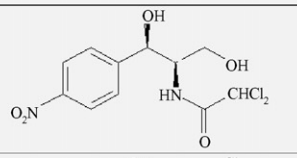
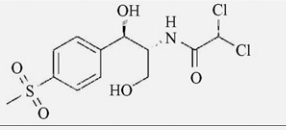
Drug	Structure	IUPAC name
AMX		4-Thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, 6-[[(2R)-2-amino-2-(4-hydroxyphenyl)acetyl]amino]-3,3-dimethyl-7-oxo
AMA		2-Thiazolidineacetic acid,α-[[2-amino-2-(4-hydroxyphenyl)acetyl]amino]-4-carboxy-5,5-dimethyl-
SDI		Benzenesulfonamide, 4-amino-N-2-pyrimidinyl
NDI		Acetamide, N-[4-[(2-pyrimidinylamino)sulfonyl]phenyl]
AMP		4-Thia-1-azabicyclo[3.2.0]heptane-2-carboxylic acid, 6-[[(2R)-2-amino-2-phenylacetyl]amino]-3,3-dimethyl-7-oxo-
APA		2-Thiazolidineacetic acid,α-[[(2R)-aminophenylacetyl]amino]-4-carboxy-5,5-dimethyl-(9Cl)
TMP		2,4-Pyrimidinediamine, 5-[(3,4,5-trimethoxyphenyl)methyl]

Table 1 (*Continued*)

SMR		Benzenesulfonamide, 4-amino-N-(4-methyl-2-pyrimidinyl)
NMR		Acetamide, N-[4-[[(4-methyl-2-pyrimidinyl)amino]sulfonyl]phenyl]
OXT		2-Naphthacenecarboxamide,4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,5,6,10,12,12a-hexahydroxy-6-methyl-1,11-dioxo-
SMZ		Benzenesulfonamide, 4-amino-N-(4,6-dimethyl-2-pyrimidinyl)
NMZ		Acetamide, N-[4-[[(4,6-dimethyl-2-pyrimidinyl)amino]sulfonyl]phenyl]
CLT		2-Naphthacenecarboxamide, 7-chloro-4-(dimethylamino)-1,4,4a,5,5a,6,11,12a-octahydro-3,6,10,12,12a-pentahydroxy-6-methyl-1,11-dioxo-
SMX		3-Quinolinecarboxylic acid,1-cyclopropyl-6-fluoro-1,4-dihydro-5-methyl-7-(3-methyl-1-piperazinyl)-4-oxo
CLF		Acetamide, 2,2-dichloro-N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)-2-(4-nitrophenyl)ethyl]
TIF		Acetamide, 2,2-dichloro-N-[(1R,2R)-2-hydroxy-1-(hydroxymethyl)-2-[4-(methylsulfonyl)phenyl]ethyl]

supernatant obtained was separated from the solid phase and a 20 μL aliquot of this solution was injected onto the HPLC system.

2.4.2. Procedure 2. Solid phase extraction (SPE)

10 μL of formic acid was added to urine samples (500 μL) before SPE procedure. The samples were extracted through a 200 mg/6 mL Bond Elut® Plexa™ cartridges (Varian Inc., Madrid, Spain). The cartridges were preconditioned with 3 mL of methanol and 3 mL of deionized water. Urine samples were then passed through the cartridges at a flow rate of approximately 1 mL/min and then rinsed with 2 mL of 1.5% NH_3 solution, 2 mL of 5% formic acid and 2 mL of aqueous solution 2% methanol prior the elution. After that, the cartridges were dried by nitrogen stream to remove excess of water and finally the analytes were eluted with 3 mL of methanol, 3 mL of

Table 2
Monitoring wavelengths and retention times.

DAD			
Analyte	λ absoprtion (nm)	Retention time (min)	SD (min) (n = 10)
AMA	230	5.46	0.005
AMX	230	6.76	0.010
SDI	280	13.69	0.002
APA	230	14.49	0.002
TMP	280	16.15	0.008
AMP	230	16.85	0.001
SMR	280	17.35	0.001
OXT	280	18.54	0.000
SMZ	300	20.15	0.001
CLT	280	24.11	0.049
SMX	280	26.99	0.005
NDI	280	29.05	0.004
CLF	280	30.15	0.002
NMR	280	31.07	0.003
NMZ	280	32.15	0.001
FLD			
	λ_{exc} (nm)	λ_{em} (nm)	
AMA	235	310	6.10 0.004
AMX	235	310	7.53 0.006
SMR	260	430	17.96 0.001
TIF	260	296	20.77 0.002
SMX	260	381	27.55 0.007
NDI	265	340	29.72 0.009
NMR	265	340	31.67 0.001
NMZ	265	340	33.06 0.003

acetonitrile and 3 mL of dichloromethane at a flow rate of 1 mL/min. The extracts so obtained were evaporated by a nitrogen stream and dissolved again with 500 μL of deionized water.

2.5. Urine samples from patients

Urine from patients under treatment with one or more of the target analytes, from whose informed consent was obtained was collected. Samples were immediately submitted to the above-described procedures in order to determine the concentration of the different analytes present in the sample.

3. Results and discussion

3.1. Chromatographic conditions

Special emphasis was devoted to the chromatographic separation due to the different chromatographic behaviour of the target drugs. Different ratios and flow-rates of the mobile phase and columns were studied in order to shorten retention times of the analytes and to improve peak symmetry with a good resolution.

The column Phenomenex® Gemini C_{18}e (150 mm) was selected as working column as good peak symmetry and resolution was achieved and it shortens retention times of the analytes which reduces time of analysis. A longer column (25 cm) yielded tailed peaks in some cases and time of analysis lengthened considerably. Other columns (LiChrospher® 100 RP-18 250 mm × 4.6 mm and Phenomenex® Prodigy ODS (3) 100 Å 250 mm × 4.6 mm I.D.) were tested but good resolution was not achieved and they also yielded tailed peaks in some cases and time of analysis lengthened considerably.

For mobile phase selection, formic acid 0.1% in water (pH 2.6) and acetonitrile or methanol were used. Both solvents gave good results in terms of resolution, peak symmetry and retention time, slightly better when using acetonitrile but this one also presents a lower absorbance at low wave lengths than methanol and this was considered an important parameter to its selection, so a combination acetonitrile:formic acid 0.1% in water in gradient elution mode was used as mobile phase.

The application of the gradient elution program described in the experimental section gave a good chromatographic separation of the compounds, with retention times values in the range from 5.46 to 33.06 min (Table 2). A higher percentage of acetonitrile shortens the chromatogram but good separation of the target analytes was not achieved properly. In contrast, when lower percentages of acetonitrile were used, the chromatographic separation was very good but the time of analysis was too long. A step of seven minutes in isocratic mode is needed to achieve the separation of AMA and AMX from the elution front.

Working temperature was chosen as the minimum possible to allow proper thermostating without sacrificing resolution and sensitivity.

Several drug-free human urine samples were mixed obtaining a pool and were processed as described above to ascertain the level of background peak interference at the elution times corresponding to the analyzed drugs. No interfering peaks were observed near the retention time of examined drugs in 10 batches of human urine pooled samples.

DAD and FLD representative chromatograms of a spiked human urine sample at a concentration level of 3 μg mL^{-1} are shown in Fig. 1A and B, blank chromatograms are showed in Fig. 1C and D. Under the chromatographic conditions described above, all the examined drugs were well separated in 33.06 min, with good peak resolutions, sharpness and symmetry. Peak purity was also corroborated by comparing UV spectra or fluorescence spectra at the peak upslope, apex and down slope of the urine samples with a standard. The chromatographic run time of 34 min was sufficient for sample analysis, which allows the analysis of a large number of samples in an acceptable period of time. The system achieved the state of equilibrium after 5 min before finishing the previous analysis.

3.2. SPE optimization

Two different solid phases were tested, a C_{18} (Discovery® DSC-18) and a Bond Elut® Plexa™. Best rates of recovery were obtained with Bond Elut® Plexa™ cartridges, their polymeric characteristics allows the extraction of acidic, neutral even basic analytes of a wide polarity range. The effect of pH was studied by adjusting the pH value of the sample at a pH 2.5, 4.0, 7.0 and 9.0; an increase in pH led to a reduction in the extraction efficiency so therefore a pH 2.5 was selected. Methanol, acetonitrile and dichloromethane were tested as elution solvents. When solvents were used alone, extraction efficiency was not satisfactorily enough even when higher volumes of solvent (10 mL) were used; only between 30% and 40% of recovery was obtained for most of the analytes. Different combinations of elution solvents were tested to improve extraction efficiency, finally a combination of 3 mL of methanol, 3 mL of acetonitrile and 3 mL of dichloromethane led to best recovery rate. Higher volumes of this solvent combination did not increase too much the extraction efficiency, so 3 mL were chosen in order to not lengthen time of analysis too.

3.3. Comparison between sample treatment procedures

Comparison between the two sample treatment methods proposed in this paper has been made in terms of extraction efficiency, time of analysis, waste of solvent and sensitivity. From the time of analysis point of view, SPE implies longer times than the simple procedure 1. Waste of solvent is very similar in both cases therefore this parameter is not determinative for the selection of either the methods. Extraction efficiency in terms of recoveries is shown in Table 3 where it can be seen that values are in general higher

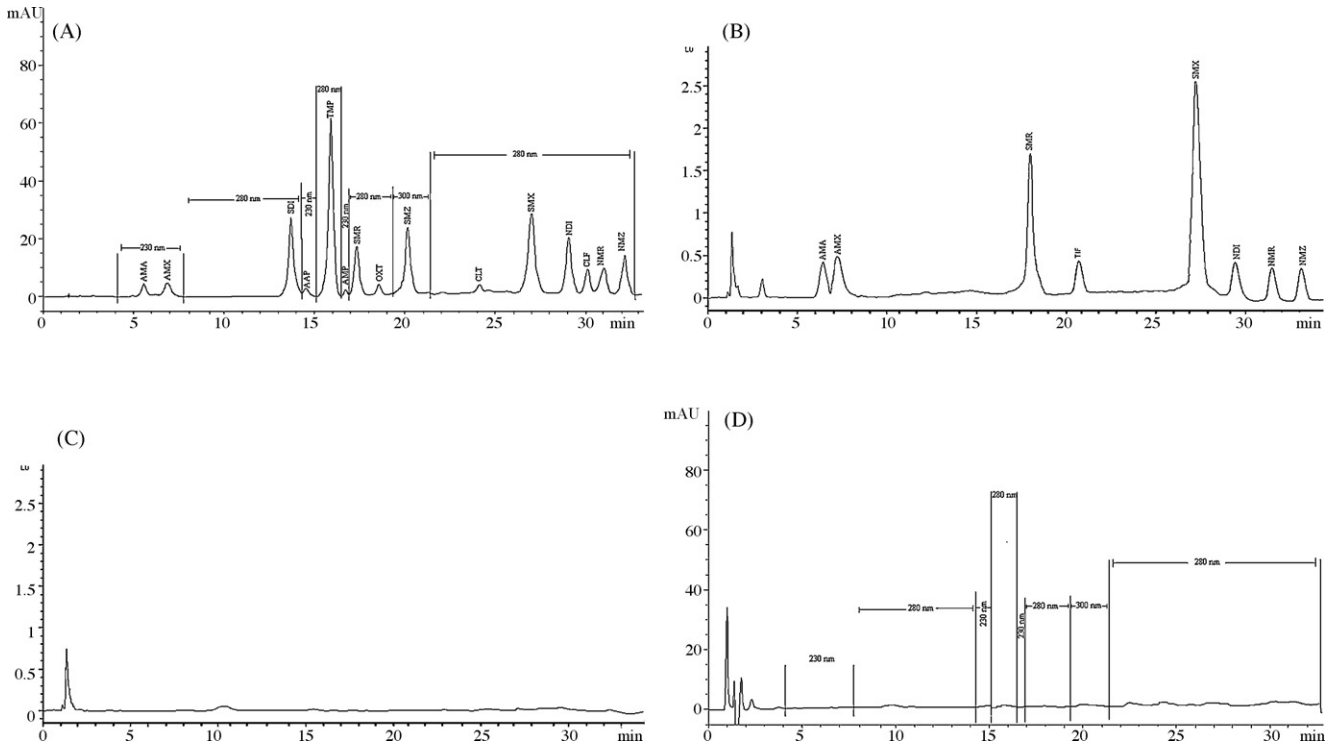


Fig. 1. Representative chromatograms obtained from a spiked urine sample containing all analytes (3 μg mL^{-1} of all standards) using procedure 1: (A) DAD detection, (B) FLD detection, (C) blank urine DAD detection, (D) blank urine FLD detection.

when the procedure 1 is applied. Finally, the parameter sensitivity was compared between both methods, in this case and as it can be seen in Table 4, SPE presents higher sensitivity because the procedure 1 implies a dilution of the sample while SPE method does not. Therefore SPE procedure would be the right selection to determine low levels of analyte. Nevertheless, levels of the drugs studied in this paper use to be very high in urine samples.

3.4. Validation study

The validation of the proposed method was done according to Gonzalez et al. [59] who give a step-by-step guide to method validation considering the most relevant procedures for checking the quality parameters of the analytical methods. We have mainly considered the items related to the estimation of the

Table 3
Recoveries ± SD in spiked urine (n = 3) at three concentration levels.

Analyte	Procedure 1			Procedure 2		
	3 μg mL^{-1}	6 μg mL^{-1}	10 μg mL^{-1}	3 μg mL^{-1}	6 μg mL^{-1}	10 μg mL^{-1}
DAD						
AMA	70.1 ± 6.4	89.1 ± 1.6	75.1 ± 5.3	46.2 ± 5.2	46.5 ± 8.4	46.2 ± 3.8
AMX	55.2 ± 3.2	56.3 ± 1.5	58.2 ± 2.0	95.5 ± 2.5	99.3 ± 8.1	99.4 ± 1.7
SDI	71.0 ± 2.4	64.7 ± 4.1	75.6 ± 2.8	36.6 ± 2.0	33.2 ± 4.9	33.3 ± 4.4
APA	71.3 ± 0.9	71.4 ± 9.0	77.4 ± 1.1	85.1 ± 1.5	81.4 ± 1.4	81.6 ± 1.0
TMP	90.2 ± 1.7	98.0 ± 1.8	97.2 ± 9.7	77.2 ± 11.9	70.2 ± 4.6	70.5 ± 1.5
AMP	80.9 ± 4.8	82.0 ± 6.9	81.8 ± 5.5	93.1 ± 5.5	87.1 ± 1.0	87.3 ± 4.6
SMR	74.6 ± 9.6	73.2 ± 1.1	74.6 ± 2.7	69.1 ± 2.8	70.6 ± 1.8	70.2 ± 1.1
OXT	94.5 ± 5.8	99.2 ± 2.0	98.6 ± 3.9	64.0 ± 1.5	62.7 ± 7.2	62.5 ± 1.2
SMZ	96.0 ± 4.6	98.3 ± 4.6	98.0 ± 2.0	96.0 ± 2.1	99.5 ± 7.3	99.3 ± 2.4
CLT	98.0 ± 4.6	99.4 ± 1.3	91.0 ± 6.8	55.0 ± 5.8	56.7 ± 4.7	56.2 ± 3.2
SMX	85.3 ± 3.7	84.7 ± 8.1	99.6 ± 2.0	90.5 ± 2.9	86.8 ± 1.5	86.4 ± 3.3
NDI	43.4 ± 0.4	45.2 ± 4.3	46.3 ± 7.5	58.4 ± 4.9	56.4 ± 1.9	56.6 ± 5.3
CLF	99.0 ± 2.0	96.1 ± 1.0	98.2 ± 1.0	86.3 ± 1.3	71.3 ± 2.3	71.9 ± 3.9
NMR	86.7 ± 5.2	93.0 ± 5.5	90.5 ± 2.9	90.4 ± 2.2	91.2 ± 2.5	91.7 ± 1.9
NMZ	41.4 ± 9.5	39.6 ± 1.4	41.0 ± 8.4	71.5 ± 2.3	74.2 ± 4.5	74.5 ± 4.7
FLD						
AMA	70.0 ± 11.4	91.4 ± 4.9	79.0 ± 2.7	46.6 ± 1.3	49.1 ± 8.5	47.2 ± 1.1
AMX	56.3 ± 2.8	58.3 ± 1.0	55.0 ± 3.9	93.6 ± 1.7	98.0 ± 3.7	96.8 ± 1.5
SMR	75.8 ± 2.7	75.7 ± 3.7	74.3 ± 1.9	70.4 ± 7.3	68.7 ± 1.5	64.4 ± 1.7
TIF	83.1 ± 6.6	83.3 ± 1.0	98.2 ± 5.1	87.8 ± 2.8	91.5 ± 2.9	96.7 ± 4.7
SMX	87.0 ± 2.1	83.2 ± 6.5	98.5 ± 1.5	91.2 ± 1.2	86.4 ± 1.9	87.6 ± 1.8
NDI	42.1 ± 1.4	47.8 ± 4.9	45.4 ± 1.6	58.3 ± 4.1	55.4 ± 3.7	57.2 ± 7.7
NMR	76.3 ± 7.1	70.9 ± 1.9	79.1 ± 2.8	88.2 ± 1.2	93.3 ± 2.5	102 ± 1.9
NMZ	47.4 ± 7.7	41.0 ± 3.6	43.0 ± 1.2	71.2 ± 5.5	74.2 ± 4.5	71.1 ± 4.9

Table 4
Methods limits of detection (LOD) and quantification (LOQ).

Analyte	Procedure 1		Procedure 2	
	MLOD ($\mu g\,mL^{-1}$)	MLOQ ($\mu g\,mL^{-1}$)	MLOD ($\mu g\,mL^{-1}$)	MLOQ ($\mu g\,mL^{-1}$)
DAD				
AMA	0.54	1.80	0.43	1.41
AMX	0.49	1.62	0.18	0.59
SDI	0.41	1.35	0.44	1.47
APA	0.38	1.26	0.18	0.56
TMP	0.35	1.17	0.18	0.57
AMP	0.60	1.99	0.11	0.36
SMR	0.35	1.17	0.18	0.57
OXT	0.65	2.16	0.37	1.25
SMZ	0.61	2.03	0.07	0.25
CLT	0.62	2.08	0.22	0.73
SMX	0.32	1.08	0.12	0.42
NDI	0.65	2.17	0.22	0.73
CLF	0.51	1.71	0.19	0.63
NMR	0.62	2.07	0.23	0.75
NMZ	0.79	2.63	0.10	0.35
FLD				
AMA	0.24	0.81	0.04	0.09
AMX	0.24	0.81	0.02	0.06
SMR	0.27	0.90	0.01	0.06
TIF	0.86	2.88	0.32	1.06
SMX	0.16	0.54	0.06	0.19
NDI	0.53	1.77	0.02	0.08
NMR	0.20	0.66	0.01	0.05
NMZ	0.45	1.53	0.01	0.06

well-known performance characteristics parameters. The following criteria were considered: selectivity, sensitivity, matrix effect linearity of the response function and accuracy (precision and trueness).

The selectivity criterion was that the analytes peaks were spectrally pure and had a baseline chromatographic resolution of at least 1.5 from all other sample components. Resolution was >1.5 for all peaks; AMA 1.64, AMX 1.64, SDI 1.55, APA 1.55, TMP 1.67, AMP 1.48, SMR 1.48, OXT 2.52, SMZ 2.52, CLT 3.5, SMX 2.7, NDI 1.62, CLF 1.98, NMR 1.81 and NMZ 1.76 for DAD detection; and AMA 1.78, AMX 1.78, SMR 5.63, TIF 5.62, SMX 2.55, NDI 2.55, NMR 2.31 and NMZ 2.52 for FLD detection. The purity of the peaks was checked by comparing UV and FLD emission spectra at the peak upslope, apex and downslope.

The potential interference of ibuprofen, paracetamol and salicylic acid (usually co-administered with this drugs) at a concentration of 30 $\mu g\,mL^{-1}$ was tested and none of them were observed under our conditions.

Linearity of the response function was studied from external calibration. A 10-point (in triplicate) calibration curve, based on peak areas, was constructed using a least-square linear regression analysis of standard mixtures of the analytes at different concentrations. A linear relationship was obtained with correlation coefficients $r \geq 0.999$ (figures depicted in Table 5) and the calibra-

Table 5
Analytical parameters of calibration.

Analyte	ILOD (($\mu g\,mL^{-1}$)	ILOQ (($\mu g\,mL^{-1}$)	Correl. coef.	Linearity of response function (%)	Linear dynamic range ($\mu g\,mL^{-1}$)
DAD					
AMA	0.20	0.65	0.9994	98.83	0.65–20.0
AMX	0.18	0.59	0.9993	98.68	0.59–20.0
SDI	0.15	0.50	0.9999	99.53	0.50–20.0
APA	0.14	0.45	0.9993	98.70	0.45–20.0
TMP	0.13	0.44	0.9996	99.13	0.44–20.0
AMP	0.11	0.36	0.9999	99.54	0.36–20.0
SMR	0.13	0.45	0.9995	98.89	0.45–20.0
OXT	0.24	0.81	0.9992	98.61	0.81–20.0
SMZ	0.07	0.25	0.9994	98.81	0.25–20.0
CLT	0.12	0.40	0.9991	98.52	0.40–20.0
SMX	0.12	0.42	0.9993	98.65	0.42–20.0
NDI	0.13	0.42	0.9991	98.52	0.42–20.0
CLF	0.19	0.63	0.9990	98.49	0.63–20.0
NMR	0.23	0.75	0.9991	98.56	0.75–20.0
NMZ	0.07	0.25	0.9994	98.49	0.25–20.0
FLD					
AMA	0.02	0.06	0.9994	98.80	0.06–20.0
AMX	0.02	0.06	0.9993	98.72	0.06–20.0
SMR	0.01	0.04	0.9997	99.28	0.04–20.0
TIF	0.32	1.06	0.9993	98.72	1.06–20.0
SMX	0.06	0.19	0.9996	99.11	0.19–20.0
NDI	0.01	0.05	0.9992	98.64	0.05–20.0
NMR	0.01	0.05	0.9998	98.37	0.05–20.0
NMZ	0.01	0.03	0.9994	98.76	0.03–20.0

tion curves obtained showed no changes over the course of one month.

Sensitivity parameters such as limit of detection and quantitation were calculated as the minimum concentration of an analyte giving peaks whose signal-to-noise ratio are 3 and 10, respectively. Instrumental limits (ILOD and ILOQ) are listed in Table 5 and method limits (MLOD and MLOQ) are listed in Table 4.

The proportional and constant biases due to possible matrix effects were evaluated following the method based on recovery assays on spiked samples. Blank urine was spiked with the analytes at three concentration levels (in triplicate) covering the concentration range of the method and subjected to the entire analytical procedure. The calibration curve obtained from the analytical signal at each addition was compared with calibration curve obtained from external calibration. According to Gonzalez et al. [59] the recovery can be calculated by the equation $R = b_{spiked}/b$ where b_{spiked} and b are the slopes of the calibration equations obtained from spiked plasma and external calibration, respectively. The absence of proportional bias corresponds to $b_{spiked} = b$, or in terms of recovery $R = 1$. This must be checked for statistical significance. Calculating $t = |R - 1|/u(R)$, where $u(R)$ is the uncertainty given by: $u(R) = \sqrt{(u^2(b_{spiked})/b^2) + (b^2_{spiked}u^2(b)/b^4)}$, where the uncertainties $u^2(b_{spiked})$ and $u^2(b)$ are obtained from the statistical parameters of the spiked and external calibration functions, if $t \leq t_{tab}$ were t_{tab} is the two-tailed tabulate value, recovery is not significantly different from 1. Alternatively, instead of t_{tab}, coverage factor (k) may be used for the comparison (at 95% confidence $k = 2$). As it can be seen in Table 6, recovery is not significantly different from 1 in general, except for AMX and AMA, when procedure 1 is applied. In these cases, t_{crit} is higher than coverage factor in DAD detection; their retention times are near the front of elution and detection of these analytes is done at 230 nm, so the matrix effect was expectable. Conversely, no matrix effect was observed in FLD detection and accordingly quantification might be carried out with.

In order to check constant bias, the intercept values of the calibration equations were tested. The absence of constant bias corresponds to an offset = 0. This must be checked for statistical significance like it was done to evaluate proportional bias. Calculating $t = |A_{spiked} - A|/u(A)$, where $u(A)$ is the uncertainty given by: $u(A) = \sqrt{S_A^2 + S_{A_{spiked}}^2}$ and A_{spiked} and A are the intercept values obtained from the calibration equations. If $t \leq 2$, offset is not significantly different from 0. Table 6 shows the results obtained and as it can be seen offset are not different from 0 in general except for AMX and AMA as in the same way that happened for proportional bias.

Intermediate precision and trueness studies can be performed using the prediction of actual concentrations from the validation standards selected for the analytical assay in the $m \times p \times n$ design (m = analytical levels, p = days and n = replications). From the corresponding ANOVA, the intermediate precision as well as the presence of significant bias is computed. To evaluate the repeatability and the intermediate precision, spiked samples ($n = 3$) at three concentrations levels 3, 6 and 10 μg mL^{-1} of each analyte were subjected to the entire analytical procedure and measured in one single day and one day per week during two months, respectively. The repeatability, obtained with DAD detection, expressed as relative standard deviation, was in the range 0.56–3.35% when procedure 1 was used and 1.01–3.32% when procedure 2 was used. The repeatability, obtained with FLD detection, was in the range 1.35–3.69% when procedure 1 was used and 1.12–3.86% when procedure 2 was used. Intermediate precision, obtained with DAD detection, also expressed as relative standard deviation, was in the range 1.51–6.61% for procedure 1 and 4.50–11.3% for procedure 2. Intermediate precision, obtained with FLD detection, was in the range 1.23–6.34% for procedure 1% and 5.50–10.5% for procedure 2. The trueness was evaluated by the bias calculation; according to Gonzalez et al. [59], a bias value δ_{pn} (p = days and n = replications) given by δ_{pn} = estimated average concentration–true value, have to be calculated from the results obtained with the validation standards.

Table 6
Matrix effect results.

Analyte	Procedure 1		Procedure 2	
	Proportional bias t_{crit}[a]	Constant bias t_{crit}[a]	Proportional bias t_{crit}[a]	Constant bias t_{crit}[a]
DAD				
AMA	9.02	4.02	0.81	0.22
AMX	19.4	2.40	0.93	0.13
SDI	0.48	1.34	0.33	0.08
APA	2.62	1.37	1.02	0.29
TMP	0.87	0.59	0.56	0.20
AMP	2.01	0.02	0.47	0.33
SMR	1.94	2.06	0.89	0.07
OXT	1.99	1.23	0.32	0.04
SMZ	1.48	2.02	0.78	0.10
CLT	1.88	0.21	1.05	0.95
SMX	1.87	1.06	1.10	0.77
NDI	0.09	1.72	0.43	0.25
CLF	2.08	2.04	0.11	0.04
NMR	0.66	2.14	1.54	0.12
NMZ	1.49	2.07	1.33	0.27
FLD				
AMA	1.74	0.87	0.48	0.02
AMX	1.39	1.35	0.93	0.05
SMR	0.25	1.53	1.02	0.24
TIF	0.21	2.02	1.22	0.95
SMX	1.67	1.37	1.59	0.66
NDI	0.20	1.78	1.78	0.48
NMR	1.49	2.09	1.23	1.02
NMZ	0.83	1.98	0.56	0.33

[a] Coverage factor $K = 2$

Table 7
Urine samples from patients results.

Analyte	Time of collection after administration	Cumulative human urine content (mg)	
		Procedure 1 ($n=3$)	Procedure 2 ($n=3$)
SMX[a]	12 h	160 ± 1.5	169 ± 3.9
TMP[a]	12 h	142.6 ± 2.7	145.3 ± 4.3
AMX[b]	6 h	280.7 ± 1.6	273.1 ± 4.9
AMA[b]	6 h	77.1 ± 2.6	75.8 ± 2.8
TIF[c]	12 h	650.2 ± 3.2	660.5 ± 5.5

[a] Cumulative urine content from an oral dose of 800 mg SMX + 160 mg TMP every 8 h.
[b] Cumulative urine content from an oral dose of 500 mg AMX every 8 h.
[c] Cumulative urine content from an oral dose of 500 mg TIF every 8 h.

Calculating $t = \delta_{pn}/u(\delta)$, where $u(\delta)$ is the uncertainty given by: $u(\delta) = \sqrt{(S_R^2(1-\gamma+(\gamma/n))/p}$, where the uncertainty S_R^2 is the intermediate precision, p is the number of days, n is the number of replicates and $\gamma = S_r^2/S_R^2$ and S_r^2 is the repeatability, if $t \leq t_{tab}$ were t_{tab} is the two-tailed tabulate value, bias is not significantly different from 0. Alternatively, instead of t_{tab}, coverage factor (k) may be used for the comparison (at 95% confidence $k = 2$). The t values obtained in all cases were under coverage factor (k) in all cases for the two procedures.

3.5. Urine samples from patients

Human urine samples from three patients under treatment with one or more of the analytes studied were collected. Table 7 shows the doses administered to patients and the values obtained by the two sample treatment procedures described in previous sections. As can be seen, results obtained by the two sample treatment procedures are in agreement.

4. Conclusion

A sensitive, selective and reproducible reversed-phase HPLC method has been developed to determine 11 drugs and their main metabolites belonging to four different families in human urine samples.

The calibration curves show linear response over the whole range of concentration used in the assay procedure. A simple reliable RP-HPLC method with satisfactory precision, accuracy and linearity has been developed and validated for simultaneous determination of the examined drugs and their main metabolites. The method has also a relatively short run time (34 min) which allows quantifying a large number of samples daily.

The proposed chromatographic system might be highly applicable in medical diagnosis. In an environment of Intensive Care Unit therapies for example, many drugs are used if patients are suffering of multi organ dysfunction syndrome. In these conditions, the therapeutic effect of many drugs may be dangerous. Moreover, the drug interactions become much more complicated. The analytical method presented in this study may help to underlying processes of drug excretion when therapeutic goals are not being achieved or even to determine if a patient is under any kind of treatment when communication is not possible; which can prevent incompatibilities between drugs.

The strength of practical application of this method is on the possibility of simultaneous determination of several drugs and metabolites in the same chromatography system and even more the determination of several drugs without changing any condition which implies saving time. In conclusions, the RP-HPLC method, developed in this study, was proven to be acceptable for drugs assay in human urine samples.

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