Please cite as: Abuzar Kabir[a,*], Kenneth G. Furton[a], Nicola Tinari[b], Laurino Grossi[c], Denise Innosa[d], Daniela Macerola[e], Angela Tartaglia[e], Valentina Di Donato[e], Cristian D'Ovidio[f], Marcello Locatelli[e,*] (2018) Fabric phase sorptive extraction-high performance liquid chromatography-photo diode array detection method for simultaneous monitoring of three inflammatory bowel disease treatment drugs in whole blood, plasma and urine

J Chromatography 2018;B 1084:53-63 doi: 10.1016/j.jchromb.2018.03.028

Fabric phase sorptive extraction-high performance liquid chromatography-photo diode array detection method for simultaneous monitoring of three inflammatory bowel disease treatment drugs in whole blood, plasma and urine

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ARTICLE INFO

Keywords:
FPSE-HPLC-PDA
Method validation
Inflammatory bowel disease
Whole blood, plasma and urine
Sample preparation

ABSTRACT

This paper reports a novel fabric phase sorptive extraction-high performance liquid chromatography-photodiode array detection (FPSE-HPLC-PDA) method for the simultaneous extraction and analysis of three drug residues (ciprofloxacin, sulfasalazine, and cortisone) in human whole blood, plasma, and urine samples, generally administered in human patients to treat inflammatory bowel disease (IBD).

The drugs of interest were well resolved using a Luna C_{18} column (250 mm × 4.6 mm; 5 μm particle size) in gradient elution mode within 20 min. The analytical method was optimized and validated in the range 0.05–10 μg/mL for whole blood, 0.25–10 μg/mL for human plasma, and 0.10–10 μg/mL for human urine. Blank human whole blood, plasma, and urine were used as the sample matrix for the method development and validation; while methyl-*p*-hydroxybenzoate was used as the internal standard (IS). Weighted-matrix matched standard calibration curves showed a good linearity up to a concentration of 10 μg/mL. The intra- and inter-day accuracy values (precision and trueness) were found in the range from −10.9% to 12.3%, and the performances of the validated FPSE-HPLC-PDA were further tested on real IBD patient samples.

This is the first FPSE procedure applied simultaneously to whole blood, plasma, and urine samples for the determination of residual IBD drugs, which possess a wide range of polarity (logP values ranging from 2.30 for Ciprofloxacin, to 1.66 for Cortisone, and 2.92 for Sulfasalazine). The new approach exhibits high potential for immediate adoptation as a rapid, robust and green analytical tool for future clinical and pharmaceutical applications.

1. Introduction

The incidence and prevalence of inflammatory bowel disease (IBD) have substantially increased in the West during past 50 years. Both Crohn's disease (CD) and ulcerative colitis (UC), the two major forms of IBD, are incurable [1]. IBD begins in young adulthood and continues throughout the lifespan. Many of the Crohn's disease patients require intestinal surgery, and some require a permanent stoma. Patients with ulcerative colitis often require colectomy. Among different treatment regimen used in IBD, therapeutic intervention by oral administration of cortisone, ciprofloxacin and/or sulfasalazine among others are common practices. Aggressive therapeutic intervention, based on the treatmentof early recurrent lesions in asymptomatic individuals, may result in effective management on the progression of these chronic diseases. Pharmacokinetic and other clinical investigations of these drugs are generally carried out using physiological samples including plasma,

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https://doi.org/10.1016/j.jchromb.2018.03.028
Received 5 February 2018; Received in revised form 13 March 2018; Accepted 15 March 2018
Available online 16 March 2018
1570-0232/

serum, urine, and saliva. These physiological samples shows prepara-tion and analysis challenges due to the presence of numerous inter- fering components in the matrix. The interferents, specially the mac-romolecules, may clog sampling and extraction devices, interact with the analytes of interest, co-elute with the target analytes and may ad- versely impact on the overall analytical performance [2]. In addition,due to the trace level concentration of the residual IBD drugs in a small volume of available physiological sample, a preconcentration me- chanism is necessary in order to obtain signal-to-noise ratios high en- ough to validate a sensible bioanalytical method. As such, a rigorous sample preparation strategy is needed that can selectively and effi- ciently isolate the target analytes from the sample matrix, eliminate/minimize the interferents and efficiently preconcentrate the analytes to a level that the analytical instrument can detect them with high con- fidence.

A number of analytical methods have been reported in the literature dealing with only one of the three drugs primarily used in IBD treat- ment that include sulfasalazine and its major metabolites in plasma using liquid-liquid extraction (LLE) followed by LC-MS/MS [3], cipro-floxacin in plasma using LLE followed by LC-MS/MS [4], in human sputum using microextraction by packed sorbent (MEPS) and HPLC- PDA [5], in plasma using LLE followed by HPLC with fluorescence detection [6], and cortisone in dry blood spot (DBS) and dry urine spot (DUS) using MEPS-HPLC [7], in urine by LLE followed by HPLC ana- lyses [8]. Due to the overwhelming difficulty in handling whole blood, most of the methods used either human plasma or urine as the in- vestigational sample matrix.

Peripheral whole blood is one of the most preferred and informa-tion-rich circulatory biofluid for pharmacokinetic, pharmacodynamics, metabolomics and other clinical investigations. However, due to the technological shortcomings of current sample preparation techniques in dealing with whole blood directly or diluted whole blood, either plasma or serum is commonly used as the primary sample matrix. Although, the implication of converting whole blood into plasma or serum in terms of potential loss of the target analytes have not been thoroughly studied. D.C. Wedge et al. reported a significant loss of metabolomic features in serum compared to plasma samples [9,10]. This observation supports a recent study performed by M. Goudarzi et al. where the researchers compared the metabolomic profile in whole blood and plasma samples after irradiating mouse to assess the impact of radiation exposure [11]. As such, it is important to highlight that if we are to take full ad- vantages of the recent progresses in hyphenated chromatographic and electrophoretic techniques, sample preparation of biofluids have to be streamlined and efforts have to be made to use original, minimally modified physiological samples for clinical investigations.

Over the last couple years, our research group has also developed a couple of bioanalytical methodologies based on plasma as the sample matrix using SPE [12], LLE [5], and MEPS [13] in combination with HPLC-PDA. In all these bioanalytical investigations, sample preparation step was found as the most challenging and critical task.

Regardless of the physicochemical characteristics of the analytes, a bioanalytical sample preparation workflow often includes a number of pretreatment steps such as protein precipitation, centrifugation, soni-cation, analyte extraction and preconcentration, elution of the ex- tracted analytes as well as post-treatment steps such as solvent eva-poration and sample reconstitution. Majority of the analytical errors and data variability stem from these steps. In clinical chemistry point of view, an ideal sample preparation technique should: (a) be capable of dealing with original or minimally modified physiological sample; (b) be biocompatible to minimize protein adsorption when exposed di- rectly to physiological samples during analyte extraction/pre- concentration; (c) eliminate the necessity of protein precipitation priorto applying analyte extraction/preconcentration step; (d) eliminate all pre-treatment and post-treatment steps from the sample preparation workflow; (e) minimize/eliminate solvent consumption during sample preparation; (f) produce a clean sample, free of protein and other interferents that potentially compromise the instrumental performance; (g) minimize the dependence on expensive chromatographic systems such as LC-MS/MS, and (f) conform to green analytical chemistry (GAC) principles.

Fabric phase sorptive extraction (FPSE), developed by Kabir and Furton [14], simplifies the analyte(s) extraction from complex matrices. FPSE has successfully addressed most of the disadvantages commonly encountered in the majority of the conventional sorbent-based extrac- tion techniques including solid phase extraction (SPE) and solid phase microextraction (SPME) [15]. FPSE is the first sample preparation technique that allows extraction of analytes directly from whole blood [11] or diluted whole blood without requiring any protein precipita- tion. The performance superiority and easiness in application of this emerging technology have already been demonstrated in a large number of research and review articles, in analyzing small drugs in biological sample matrices [16], wide variety of pollutants in en- vironmental samples [17–24], biological samples [16,24–27], for food safety studies [28,29] and in pharmaceutical analysis [30]. All these articles have unequivocally demonstrated that FPSE is a simple, rapid, and green approach that substantially simplifies the overall sample preparation workflow and substantially decreases the consumption of hazardous and toxic organic solvents. However, most of these works utilized large sample volumes (approx. 10–30 mL) that are not suitable as far as physiological samples such as whole blood are concerned (generally sample volume is limited to 0.1–1 mL).

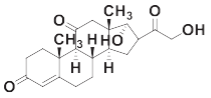
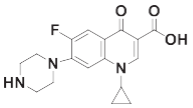
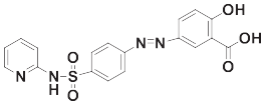
Following our previous works [5,12,13,31,32], where innovative extraction/sample preparation procedures were applied to plasma and urine samples prior to conventional, rugged, and common chromato-graphic instrument configurations frequently used in clinical applica-tions, in the current study, FPSE has been applied to whole blood, plasma and urine for simultaneous separation and analysis of cipro- floxacin, sulfasalazine, and cortisone. Particularly, among others, one goal was to demonstrate that without the availability of powerful, complex and high-end analytical instrument configurations such as LC- MS/MS that is unaffordable by many clinical laboratories, requires highly trained personnel and involves excessive maintenance costs [33,34], it is possible to obtain similar analytical performances with a so called low-end instrumentation such as HPLC-PDA. The proposed simplification in both the sample preparation and the chromatographic instrument configuration would streamline the multi-billion dollar clinical chemistry practices, minimize the usage of toxic and hazardous organic solvent, substantially reduce the overall cost for analysis, har- monize the standard practice, and reduce the lab-to-lab data variability due to the lack of harmonization of sample preparation process. Fol-lowing these aims, the current study is focused to: (1) develop a simple, robust and green analytical methodology capable of simultaneously monitoring three IBD drug residues in blood, plasma, and urine; (2) demonstrate that whole blood can be used as the investigational sample matrix without applying any protein precipitation prior to target ana- lytes extraction; (3) exhibit that solvent evaporation and sample re- constitution can be conveniently eliminated from the sample prepara- tion workflow; (4) demonstrate that a robust and effective sample preparation technique may offer cleaner sample that can be analyzed in inexpensive chromatographic systems without requiring highly ex-pensive instrument such as LC-MS/MS.

2. Materials and method

2.1. Chemicals, solvents and devices

Ciprofloxacin, methyl-*p*-hydroxybenzoate (methylparaben, IS), sul-fasalazine, and cortisone (> 98% purity grade), sodium phosphate monobasic and sodium phosphate dibasic (> 99% purity grade) and phosphoric acid were purchased from Sigma-Aldrich (Milan, Italy). Acetonitrile (AcN) and methanol (HPLC-grade) were purchased from Carlo Erba (Milan, Italy) and were used without further purification.

Table 1
Molecular structures and other pertinent physiochemical properties of selected IBD drugs.

Drug name	CAS no.	Molecular weight (g/mol)	Molecular structure	LogP
Cortisone	53-06-5	360.44		1.66
Ciprofloxacin	85721-33-1	331.34		2.30
Sulfasalazine	599-79-1	398.39		2.92

The water (18.2 MΩ·cm at 25 °C) for HPLC analysis was generated by a Millipore Milli-Q Plus water treatment system (Millipore Bedford Corp., Bedford, MA, USA).

2.2. Stock solution, calibration curve and quality control analysis

The stock solutions of chemical standards were made at the concentration of 1 mg/mL in the mobile phase. The combined working solutions (concentration range: 1–200 μg/mL) were prepared by dilu- tion of a mixed solution. The matrix-matched calibration standards and quality control samples (QCs) were prepared and analyzed as reported in Section 2.3 and Section 2.5. The resulting samples were injected in HPLC-PDA instrument.

2.3. Whole blood, plasma and urine sample collection, storage, and preparation

Whole blood, plasma and urine samples used as the blank sample matrices were collected from healthy volunteers. Volunteers were previously informed about the experimental procedures and the nature of the study, and were asked to provide written consents before the experiments. The whole blood samples were collected into heparinized tubes and used as received and also for the recovery of plasma; while urine samples were collected into sterilized containers. Whole blood, collected into heparinized tubes (green tops), was centrifuged for 10 min at 1000–2000 ×*g* using a refrigerated centrifuge, in order to remove cells and particulates from plasma.

All samples were stored at −20 °C prior to the analysis, except whole blood that was stored at +4 °C. A 180 μL aliquot of human blank whole blood was mixed with 10 μL of analyte working solutions and 10 μL of IS (100 μg/mL), diluted in the ratio 1:5 (v:v) using MilliQ water, and was vortexed for 3 min. A 450 μL aliquot of human blankplasma was mixed with 25 μL of analyte working solutions and 25 μL ofIS (100 μg/mL), and was vortexed for 3 min. A 900 μL aliquot of human blank urine was mixed with 50 μL of analyte working solutions and 50 μL of IS (100 μg/mL), and was vortexed for 3 min. All samples were then submitted to the FPSE procedure.

Methyl-*p*-hydroxybenzoate was used as the internal standard due to its intermediate logP value (1.96) compared to the selected IBD drugs.

2.4. Apparatus and chromatographic conditions

Analyses were performed using an HPLC Thermo Fisher Scientific liquid chromatography system (Model: Spectra System P2000) coupled to a photodiode array detector (PDA Model: Spectra System UV6000LP). Mobile phase was directly *on-line* degassed by using a Spectra System SCM1000 (Thermo Fisher Scientific, Waltham, MA, USA) was used to collect and analyze data. The Luna C18 (250 × 4.6 mm, 5 μm particle size; Phenomenex, Torrance, CA, USA) packing column connected to a Security Guard column (4.0 × 3.0 mm,5 μm particle size; Phenomenex, Torrance, CA, USA) was used to se- parate drugs and IS. The columns were thermostated at 25 °C (± 1 °C) using a Jetstream2 Plus column oven during the analysis. Drugs and IS were detected at the maximum wavelengths of 283 nm (ciprofloxacin), 369 nm (sulfasalazine), 260 nm (methyl-*p*-hydroxybenzoate), and 247 nm (cortisone), respectively.

The HPLC system was optimized to obtain better signal-to-noise ratio of drugs in a single chomatographic analysis, the best peak shape,an appropriate run-time, and better peak resolution. The gradient elu- tion using phosphate buffer (pH 3.5, 40 mM) as solvent A and acet- onitrile as solvent B was selected due to an interference peak present during the analyses of blank matrices, as reported in Section 3.1, andthe optimum separation of analytes were obtained using the following gradient program: 0–3 min 80% of solvent A, 3–4 min from 80% to 70% of solvent A, 4–12 min 70% of solvent A, 12–13 min from 70% to 80%of solvent A, 13–20 min 80% of solvent A (re-equilibration step). The chromatographic conditions reported herein allow to resolve the analytes with better baseline separation (*Supplementary material, sectionS.1*).

2.5. Preparation of fabric phase sorptive extraction media and developing the extraction procedure

The wide dispersion of logP values of the target analytes rangingfrom 2.30 for ciprofloxacin, to 1.66 for cortisone, and 2.92 for sulfa- salazine (Table 1) pose great challenge for their simultaneous extrac- tion and analysis from physiological sample matrices. As such, during the method development, five different FPSE media coated with dif- ferent sorbent chemistries were investigated in order to select the most efficient sorbent coating that provides the maximum enrichment factors for all the target drugs. Subsequently, the method was optimized di- rectly in different physiological sample matrices, such as whole blood, plasma and urine. The validation procedure was carried out following International guidelines [35–37] in order to demonstrate the viabilityof the method as a valuable analytical tool for clinical and pharma- ceutical applications.

The FPSE media, sol-gel Carbowax 20 M (sol-gel CW 20 M, highly polar sorbent possessing poly(ethylene glycol), $H[OCH(CH_3)CH_2]_nOH$ as the building block); sol-gel polycaprolactone-*block*-poly- dimethylsiloxane-*block*-polycaprolactone (sol-gel PCAP-PDMS-PCAP, medium polar sorbent containing poly(caprolactone), $(C_6H_{10}O_2)_n$ and poly(dimethylsiloxane), $(CH_3)_3SiO[(CH_3)HSiO]_nSi(CH_3)_3$ building

blocks, sol-gel sucrose (sol-gel SCS, highly polar possessing sucrose, $C_{11}H_{22}O_{11}$ as the building block); sol-gel poly(caprolactone) (sol-gel PCL, medium polar, containing poly(caprolactone), $(C_6H_{10}O_2)_n$ as the

building *block*) and sol-gel poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (sol-gel PEG-PPG-PEG, medium polar possessing poly(ethylene glycol), (-CH_2CH_2O-) and poly(propy- lene glycol), $H[OCH(CH_3)CH_2]_nOH$ as the building blocks). The pre- paration of the cellulose fabric substrate for sol-gel coating, the pre- paration of the sol solution for sol-gel coating and the sol-gel immersion coating process have been described in detail elsewhere [15]. Due tothe difference in organic polymer or macromer (sugar) used in the sol solution, the sorbent loading varied widely during the coating process.

For example, sol–gel Carbowax® 20 M (sol–gel CW 20 M) media ob- tained 8.63 mg/cm^2 sorbent loading; sol–gel polycaprolactone (sol–gel PCL) obtained sorbent loading of 5.23 mg/cm^2, sol–gel poly-caprolactone-*block*-polydimethylsiloxane-*block*-polycaprolactone (sol–gel PCAP-PDMS-PCAP) obtained sorbent loading of 6.14 mg/cm^2,sol-gel sucrose (sol-gel SCS) obtained sorbent loading of 4.31 mg/cm^2,and sol-gel poly(ethylene glycol)-*block*-poly(propylene glycol)-*block*-poly(ethylene glycol) (sol-gel PEG-PPG-PEG) obtained sorbent loading of 5.68 mg/cm^2 under identical coating conditions. It is important to note that unlike physical surface coating process, sol-gel immersion coating process is driven by highly controllable chemical network generation process and therefore, is highly reproducible.

Subsequently, the sol-gel sorbent coated FPSE media were cut into circular disks using a homemade puncher with internal diameter of 1 cm (surface area of 0.785 cm^2) and 0.6 cm (surface area of 0.2826 cm^2). In this way, all membranes possessed identical surface area. Particularly, the new approach of cutting the circular disks en- sures better reproducibility and standardize the system dimentions. After the cut, the FPSE membrane underwent a series of sequential steps: i) cleaning with 2 mL of acetonitrile: methanol (50:50, v:v) for5 min; ii) rinsing 2/3 times in MilliQ water holding with a tweezer; iii) sample extraction: biological matrix sample (volumes are reported inSection 2.3) at TAAB rotator for 30 min; iv) elution/back extraction using 150 μL of methanol for 10 min; v) centrifuge the eluent at 12.000 ×*g* for 10 min; vi) 20 μL of the supernatant was injected into HPLC-PDA instrument.

2.6. Method validation

The validation of analytical method was carried out according to the International Guidelines [35–37] in order to check LODs, LOQs, line- arity, intra- and inter-day trueness and precision, selectivity, recovery, stability and parallelism test of different drugs in whole blood, plasma, and urine samples.

3. Results and discussion

3.1. Optimization of HPLC separation

Following the data obtained during HPLC method development (Fig. 1), the best HPLC conditions were found as: phosphate buffer (40 mM, pH 3.5) and acetonitrile; isocratic conditions (70:30, v:v); flow rate 1 mL/min; temperature 35 °C (± 1 °C); HPLC column Luna C18 (250 mm × 4.6 mm, 5 μm).

Unfortunately, the previously reported isocratic conditions, very useful in order to avoid the challenges encountered during method transfer, could not be applied when physiological sample matrices were tested. Following optimized extraction procedure, in fact, could be observed an interfering peak at the ciprofloxacin retention time. For this reason, it was necessary to change the conditions from isocratic to gradient elution, maintaining the same mobile phase that had demon- strated better signal-to-noise values and peak shapes. Based on the isocratic experimental data, was optimized the following gradient program: 0–3 min 80% of solvent A, 3–4 min from 80% to 70% of solvent A, 4–12 min 70% of solvent A, 12–13 min from 70% to 80% of solvent A, 13–20 min 80% of solvent A (re-equilibration step). As pre- viously reported, the plateau at 70% of solvent A allowed to obtain higher chromatographic performances, for this reason, was added a preliminary step at 80% of solvent A in order to retard the ciprofloxacin peak elution and its separation/resolution from the interfering peak observed in real matrix analyses. Using this gradient elution, all the analytes were eluted in 70% of solvent A that resulted in better se- parations and no interfering peaks were observed (*Supplementary material, Section S.2 to S.4*).

3.2. Selection of FPSE sorbent chemistry and optimization of FPSE procedure

FPSE has been recently introduced and developed to extract the analytes directly from the complex sample matrices by direct absorp- tion on the extraction media without earlier sample pretreatment like protein precipitation [27]. Nevertheless, in order to enhance the ex- traction and clean up procedures from human whole blood, plasma, and urine samples using FPSE media, several experimental FPSE parameters need to be optimized, such as the most suitable sorbent type, FPSE media dimensions, sample volume, and back extracting solvent type and its volume.

In this study, all the FPSE media tested are generally recommended for non-ionizable polar and non-polar analytes, but in this work the main objective was to develop an extraction procedure for molecules that are characterized by a wide range of logP. Within the initially se- lected five membranes, the sol-gel PCAP-PDMS-PCAP and sol-gel CW 20 M coated FPSE media, showed better enrichment factors (Table 2) at three different concentration levels (0.25, 1, and 5 μg/mL). These tests were conducted with aqueous standard solutions and 1 mL of sample volume following the general procedure outlined here: (i) cleaning with 2 mL of acetonitrile:methanol (50:50, v:v) for 5 min; (ii) rinsing with 2 mL of MilliQ water for 5 min; (iii) sample extraction: 1 mL of standard solution sample at TAAB rotator for 30 min; (iv) elution/back extrac- tion using 500 μL of methanol for 10 min; (v) centrifuge at 12.000 ×*g* for 10 min; (vi) injecting 20 μL of the supernatant into the HPLC-PDA instrument. Enrichment factors (%) obtained for these five membranes are reported in Table 2.

Based on these results, sol-gel PCAP-PDMS-PCAP (M1) and sol-gel CW20M (M4) were selected for further optimization. Fig. 2 demon- strates the schematic representation of sol-gel PCAP-PDMS-PCAP and sol-gel CW 20 M sorbent coated on cellulose fabric substrate.

In fabric phase sorptive extraction process optimization, particularly we focused our attention to sample volume and back extracting solvent volume.

The back extracting solvent volume is another critical parameter that needs to be optimized in order to enhance the signal-to-noise ratio. Different volumes of methanol were tested: 50, 100, 150, and 300 μL. The best performance in back-extraction was obtained using 150 μL of methanol, obtaining the enrichment factors reported in Table 3. In order to further improve the analytical performances, the back extrac- tion with high volume of methanol (500 μL) were also evaluated fol- lowed by evaporating the solvent to dryness, reconstituting with lower solvent volumes (50 μL) and finally injecting into the HPLC system. In this case, no signal improvement was observed in the HPLC chroma-togram.

The FPSE-HPLC-PDA procedure was primarily developed and opti- mized, both for extraction and instrumental point of view, using aqu- eous standard samples. After selecting the optimum conditions using standard solutions, optimization was further accomplished using blank human whole blood, plasma, and urine sample matrices spiked with ciprofloxacin, sulfasalazine, cortisone, and internal standard, but no more improvements were observed varying the parameters previously reported for FPSE procedure. Additionally, as previously observed [16], using FPSE media the preliminary proteins precipitation step is not required because the back-extraction organic solvent not only recover the analytes from the FPSE media and adhered protein molecules, but it also concurrently performs protein precipitation. Thus, FPSE allows to

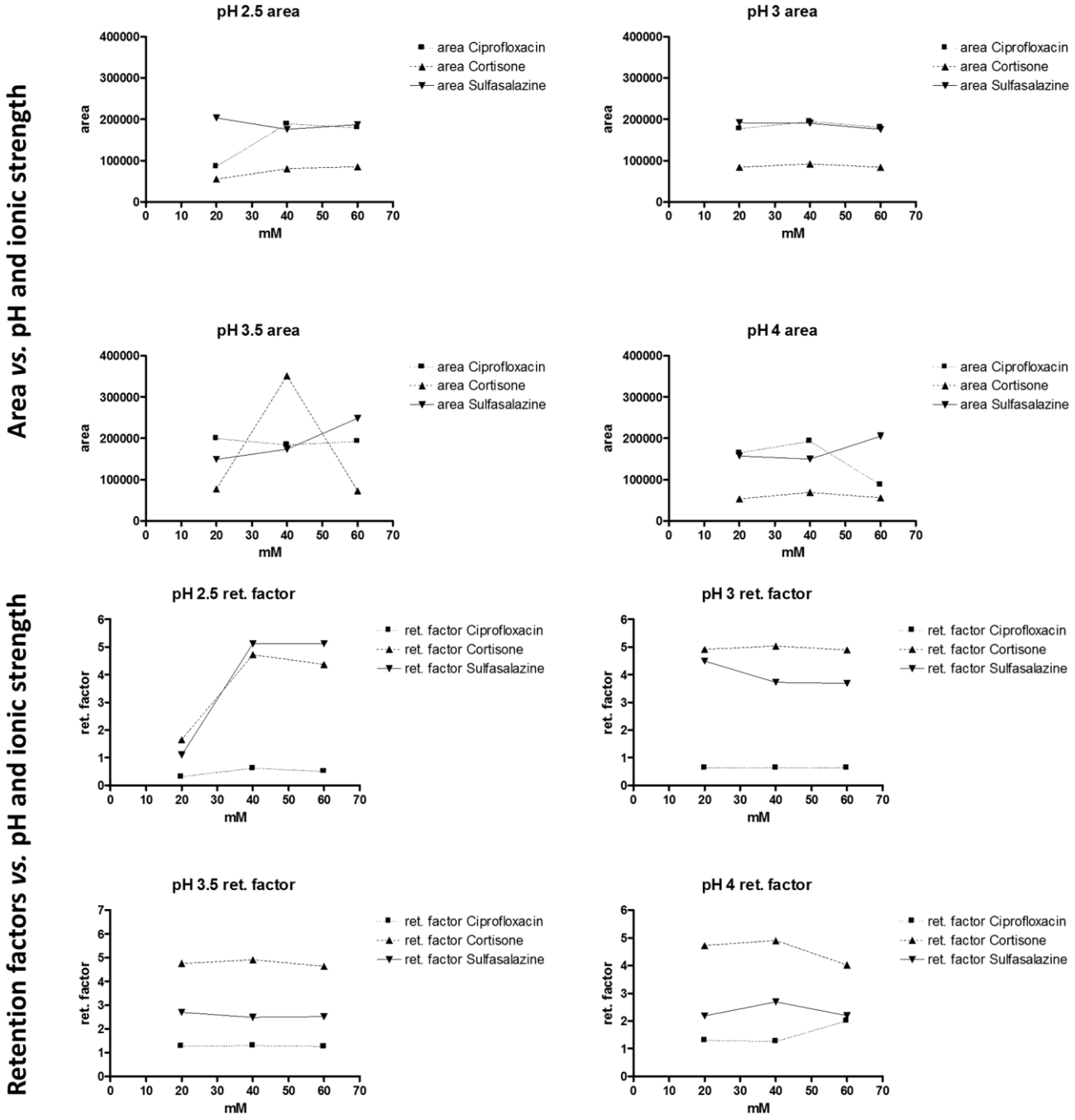


Fig. 1. Optimization of different HPLC parameters.

minimize the overall sample preparation time and reduce the solvent consumption. Also, this volume is sufficient for centrifugation (10 min at 12.000 rpm) so that a protein and particulates free supernatant canbe obtained for the downstream HPLC analysis. Only the whole blood sample matrix (see *Supplementary materials, section S.5*) was minimally modified by diluting with MilliQ water (1:5, v:v) in order to reduce the matrix density/solution viscosity and to enhance the analytes mobilities into the system from sample solution to FPSE media, as also reported in literature for large molecules [38]. Plasma and urine samples didn't undergo any modification.

The final optimized conditions, obtained using sol-gel CW 20 M media, were: i) cleaning with 2 mL of acetonitrile: methanol (50:50, v:v) for 5 min; ii) rinsing 2/3 times in MilliQ water holding with a tweezer; iii) sample extraction: biological matrix sample (volumes are reported in Section 2.3) at TAAB rotator for 30 min; iv) elution/back extraction using 150 μL of methanol for biological samples for 10 min; v) centrifuge at 12.000 ×*g* for 10 min; vi) 20 μL of the supernatant was injected into HPLC-PDA instrument.

The FPSE media can be reused up to approx. 30 times with no appreciable carry-over and no efficiency loss by washing with 2 mL acetonitrile:methanol (50:50, v:v) for 5 min. Then the media could be dried and stored in an hermetically sealed glass mainfold for reuse.

3.3. Selectivity in FPSE media

The selectivity of the extraction sorbent towards the target analytes is an important criteria for selecting a sample preparation techniques from a large number of available techniques along with their sorbent chemistries. Classical sample preparation techniques such as solidphase extraction and microextraction by packed sorbent traditionally

Table
Enrichment factors (%) observed at three different concentration level of water standard solutions. The enrichment factors were calculated as the percentage of peak area enhancement with respect to the area of reference standard solutions. The efficiencies are evaluated on 2 different membrane dimensions: 0.6 cm and 1 cm as diameter. In bold are reported the better values.

Enrichment factors (%)					Enrichment factors (%)				
Concentration 0.25 μg/mL - diam. 0.6 cm					Concentration 0.25 μg/mL - diam. 1 cm				
	Ciprofloxacin	Sulfasalazine	Methylparaben (IS)	Cortisone		Ciprofloxacin	Sulfasalazine	Methylparaben (IS)	Cortisone
M 1	8.63	39.10	12.38	11.32	M 1	14.65	80.36	26.90	31.49
M 2	14.71	49.98	33.93	16.74	M 2	28.70	64.34	52.08	23.98
M 3	14.06	59.69	36.06	46.75	M 3	21.54	58.60	48.61	7.25
M 4	13.09	35.13	46.72	45.44	M 4	28.34	74.37	46.10	58.94
M 5	9.95	58.33	33.82	18.73	M 5	27.55	62.30	48.00	37.17
Enrichment factors (%)					Enrichment factors (%)				
Concentration 1 μg/mL - diam. 0.6 cm					Concentration 1 μg/mL - diam. 1 cm				
	Ciprofloxacin	Sulfasalazine	Methylparaben (IS)	Cortisone		Ciprofloxacin	Sulfasalazine	Methylparaben (IS)	Cortisone
M 1	7.13	46.01	15.85	14.72	M 1	9.88	7.19	21.28	17.91
M 2	12.07	36.91	35.01	12.18	M 2	26.21	5.52	52.73	23.37
M 3	9.01	55.25	41.42	49.07	M 3	11.78	6.07	29.43	26.42
M 4	6.76	48.08	38.65	28.80	M 4	15.48	5.06	35.62	29.95
M 5	6.42	33.20	33.28	20.70	M 5	15.73	6.36	35.35	28.54
Enrichment factors (%)					Enrichment factors (%)				
Concentration 5 μg/mL - diam. 0.6 cm					Concentration 5 μg/mL - diam. 1 cm				
	Ciprofloxacin	Sulfasalazine	Methylparaben (IS)	Cortisone		Ciprofloxacin	Sulfasalazine	Methylparaben (IS)	Cortisone
M 1	1.82	60.81	14.56	6.88	M 1	3.54	136.23	28.72	13.37
M 2	5.88	43.46	21.14	7.45	M 2	12.22	77.19	30.75	10.16
M 3	2.83	45.59	53.04	41.12	M 3	5.71	171.65	58.40	39.45
M 4	2.49	29.88	52.83	27.35	M 4	5.95	88.30	52.52	30.42
M 5	2.48	76.42	52.39	15.23	M 5	6.57	120.73	70.13	25.08

M1: sol-gel PCAP-PDMS-PCAP; M2: sol-gel SUCROSE; M3: sol-gel PCL; M4: sol-gel CW 20 M; M5: sol-gel PEG-PPG-PEG. Analytes were eluted using 500 μL methanol.

utilize small chain alkyl ligands *e.g.*, C8, C18 ligands immobilized on silica particles. On the other hand, solid phase microextraction utilize organic polymers such as polydimethylsiloxane immobilized on a fused silica glass rod. As such, these techniques offer poor affinity towards the target analytes. FPSE, on the other hand, utilize biocompatible organic polymer such as poly(ethylene glycol), inorganic precursor such as methyl trimethoxysilane as well as hydrophilic natural polymer cellulose fabric that synergistically compliment each other to determine the overall selectivity and extraction sensitivity of the FPSE media. Therefore, FPSE enjoys freedom to fine tune the selectivity of the device as all three of its selectivity components (organic polymer, inorganic precursor, and hydrophilic/hydrofobic substrate) can be changed with suitable alternative. FPSE is the first sample preparation technique that actively utilize the surface chemistry (hydrophilic/neutral/hydro- phobic) of the fabric substrate that determines the overall selectivityand extraction affinity of the FPSE device.

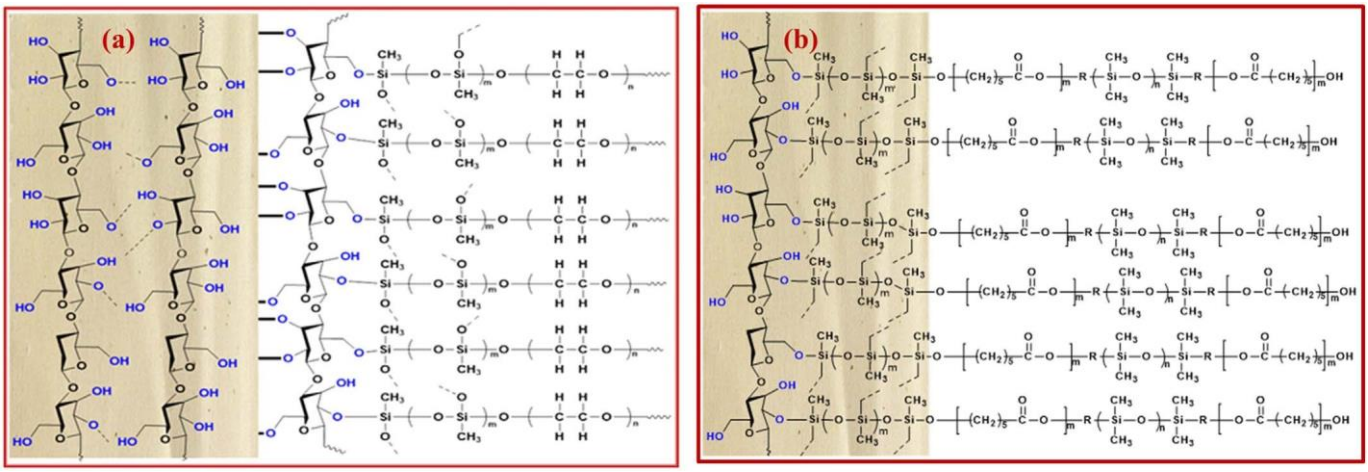


Fig. 2. Schematic representation of (a) sol-gel CW 20 M and (b) sol-gel PCAP-PDMS-PCAP coated FPSE media.

Table
Enrichment factors (%) observed at one concentration level (1 μg/mL) of water standard solutions. The enrichment factors were calculated as the percentage of peak area enhancement with respect to the area of reference standard solutions. The efficiencies are evaluated on membranes with 1 cm as diameter, sample volume of 500 μL, back extracting solvent volume of 150 μL. In bold are reported the better values.

	Ciprofloxacin		Sulfasalazine		Methylparaben (IS)		Cortisone	
	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.	Mean	Std. dev.
M 1	25.8	3.7	63.9	7.9	144.9	15.6	26.9	1.7
M 4	29.1	4.2	56.7	12.3	441.0	8.7	105.4	13.7

M1: sol-gel PCAP-PDMS-PCAP; M4: sol-gel CW 20M.

3.4. Biocompatibility of FPSE media

One major selection criteria of an appropriate sample preparation technique for physiological sample is its biocompatibility. Biocompatibility of a sample preparation technique is generally assessed by its tendency towards protein adsorption and platelet adhesion when exposed to physiological fluid, specially whole blood or plasma. However, most of the conventional sample preparation techniques are not biocompatible. Therefore, protein precipitation is often required prior to analyte extraction in order to prevent clogging or irreversible adhesion to the surface of the extraction sorbent. Both the selected sorbents in the current study, sol-gel CW 20M (polyethylene glycol) [39] and sol-gel PCAP-PDMS-PCAP [40] are biocompatible. Due totheir least interaction with the matrix protein and platelet, their extraction performances remain virtually uncompromized when exposedto whole blood or other physiological samples.

3.5. Mechanism of extraction in FPSE

FPSE has combined two major sample preparation techniques: SPE (governed by exhaustive extraction principle) and SPME (governed by equilibrium driven extraction principle) into a single sample preparation technique. Due to the geometrical advantage and the combination of both equilibrium and exhaustive extraction mechanism [15], FPSE is capable of performing exhaustive extraction under equilibrium extraction conditions. For analyte extraction and preconcentration, typically a small piece of clean FPSE medium is introduced into the sample vial containing the unmodified, original sample. A Teflon coated magneticstirbar is often used to diffuse the sample. Alternately, an orbital shaker can be used. At the end of the extraction, the FPSE medium is withdrawn from the extraction vial and is placed into the desorption tube. Depending on the size of the FPSE medium, 50–250 μL of organic solvent can be used for back-extraction/elution. Subsequently, the eluent is centrifuged or filtered and an aliquot of the clean supernatant is directly injected into the chromatographic system.

Fabric phase sorptive extraction utilizes a piece of cellulose fabric as the substrate, on the surface of which an inorganic-organic hybrid sorbent coating is created by *in situ* sol-gel immersion coating process. Sol-gel coating technology, during the *in situ* coating process, chemically binds the organic-inorganic hybrid sorbent network to the fabric substrate. The fabric substrate possesses throughpores for ventilation. The pores in the fabric remain intact even after the sol-gel surface coating process. The sponge-like porous architecture of the sol-gel sorbent, permeability of fabric support and the strong capillary action of cellulose fabric promote rapid diffusion of sample into the sol-gel extraction sorbent and consequently, enhance both the extraction and back-extraction kinetics. The rapid diffusion of the sample using a small bar magnet or an orbital shaker compels the sample containing the target analytes permeating through the sponge-like sol-gel sorbent distributed as a thin film over the fabric substrate. The sample easily passes through the thoughpores of the FPSE media. Hydrophilic property of the cellulose fabric works as a bait to bring the aqueous physiological sample towards the FPSE media for rapid analyte-sorbent interaction, leading to successful interaction. Unlike solid phase extraction where a sample passes through the extraction bed only once, in FPSE, the sample recirculates through the porous FPSE media hundreds of time during the extraction operation and consequently results in exhaustive extraction. As such, even though FPSE is generally carried out under equilibrium extraction mode, it mimics SPE like flow-through system. FPSE media can also be used as a SPE disk as demonstrated by S. S. Lakade et al. [20]. At the end of the analyte extraction, a small volume of organic solvent (50–250 μL) can efficiently elute the extracted analyte(s), thus eliminate the necessity of solvent evaporation and sample reconstitution from the sample preparation workflow.

3.6. FPSE-HPLC-PDA method validation

The optimum conditions were subsequently applied to validate the method and to the analyses of real samples from patients affected by IBD disease (like *e.g.* ileo-colic Crohn's disease) and under treatment with the considered drugs to better modulate the therapy following the concept of the therapeutic drug monitoring (TDM).

The matrix-matched calibration curves were obtained by analyzing non-zero concentration standards six-times made in freshly spiked physiological sample matrices (Table 4). By plotting the analyte/IS area ratio for each level of quantification *versus* the nominal concentration of each standard solution, was evaluated the linearity of the method (intercept, slope, determination coefficient and variation) in the range from 0.05 to 10 μg/mL for whole blood (see *Supplementary materials, section S.6*), from 0.25 to 10 μg/mL for human plasma (see *Supplementary materials, section S.7*), and from 0.10 to 10 μg/mL for human urine (see *Supplementary materials, section S.8*), and the results are reported in Table 5. The curves show a linear correlation over the range tested and determination coefficients (r^2) ≥0.9806, using a weighting factor of ($1/x^2$) according to international validation guidelines [36]. The precision (RSD%) and trueness (Bias%) values were below 15% (Table 5). The limit of quantifications were 0.05 μg/mL for human whole blood, 0.25 μg/mL for human plasma, and 0.10 μg/mL for human urine. Limit of detections were set at 0.02 μg/mL for whole blood, 0.10 for human plasma, and 0.03 μg/mL for human urine based on signal-to-noise ratios. The selectivity experiment was performed using six blank sample matrices, collected from different controls, according to ICH guideline [35]. The blank samples showed neither area values over 20% of LOQ areas at the analyte retention times, nor over 5% of IS area at the drug retention time. No significant changes of drug amount due to their potential degradation during the analysis, were carried out for stock solutions, spiked samples and extracts stored at room temperature. The spiked samples stored at −20 °C, at +4 °C, and room temperature (25 °C) samples were stable for at least 15 days. Additionally, was tested the stability of FPSE media stored under vacuum at 4 °C after the extraction process from whole blood matrix as reported in *Supplementary materials, section S.9*. This evaluation could be interesting, especially related to the possibility to extract the sample directly using FPSE media, store it and analyze it after several days, straight without further purifications and/or sample manipulations, but just the back-extraction procedure.

For the period of the study, the FPSE media were stored at 4 °C and the other samples were found stable and no degradation was observed.

Table 4
Mean linear calibration curve parameters performed by weighted-linear least-squares regression analysis of six independent eight non-zero concentration points in plasma and urine samples.

Matrix	Analyte	Linearity range (μg/mL)	Slope[a]		Intercept[a]		r^2	Wavelengths (nm)	Retention times (min)	
			Mean	Std. dev.	Mean	Std. dev.			Mean	Std. dev.
Whole blood	Ciprofloxacin	0.05–10 (0.02 μg/mL)[b]	0.02446	0.0008403	0.004699	0.0001213	0.9825	283	5.96	0.07
	Sulfasalazine	0.05–10 (0.02 μg/mL)[b]	0.01777	0.0004220	0.004142	0.000051762	0.9865	369	11.10	0.09
	Cortisone	0.05–10 (0.02 μg/mL)[b]	0.1031	0.002853	0.004067	0.0003451	0.9886	247	15.38	0.08
Plasma	Ciprofloxacin	0.25–10 (0.1 μg/mL)[b]	0.099507	0.003032	0.023070	0.001609	0.9925	283	6.09	0.09
	Sulfasalazine	0.25–10 (0.1 μg/mL)[b]	0.05358	0.001847	0.001226	0.0009805	0.9853	369	10.72	0.10
	Cortisone	0.25–10 (0.1 μg/mL)[b]	0.15460	0.004766	0.02065	0.002530	0.9825	247	15.39	0.07
Urine	Ciprofloxacin	0.10–10 (0.03 μg/mL)[b]	0.02776	0.0008704	0.02444	0.0002429	0.9931	283	5.99	0.09
	Sulfasalazine	0.10–10 (0.03 μg/mL)[b]	0.1233	0.003967	−0.002911	0.0008678	0.9806	369	10.73	0.10
	Cortisone	0.10–10 (0.03 μg/mL)[b]	0.07400	0.001595	0.006329	0.0003731	0.9808	247	15.27	0.03

[a] Values at 95% confidence intervals on the mean of six independent calibration curves.
[b] The round brackets show the LOD values obtained from signal-to-noise ratio (3); the slope and intercept of calibration curve are expressed in μg/mL.

3.7. Comparison of the current method with methods reported in the literature

From the data reported in the literature, there is not a single method that can simultaneously analyze all the three IBD drug residues in the three physiological sample matrices. In addition to the conventional plasma and urine samples, in the present study, whole blood has been used as a physiological sample matrix, which is difficult to use in the validation procedures due to the fact that it is an overly complex sample matrix.

Fig. 3 represents a schematic diagram representing the steps involved in classical sample preparation techniques, SPE and MEPS as well as the newly developed FPSE. It is worthy to note that the steps in sample preparation should not be the major selection criteria for a sample preparation technique. The selection criteria should also include chemical selectivity, ease in operation, available sorbent chemistries, greenness of the approach, pH stability of the sorbent, biodegradibility and reusability of the sample preparation technique, analyte retention capacity, minimum operational skill requirement, ability to remove matrix interferents, among others. In this regard, FPSE easily outperforms conventional sample preparation techniques, in particular, for bioanalysis.

In addition to comparing steps involved in sample preparation techniques, comparison can also be carried out using methods for individual analytes, or analytes of interest and other compounds like metabolites and/or degradation products. In literature, there are methods that reports lower limits of quantification, or involves with the use of structurally similar analytes, while in the current work a compromise was needed to optimize the simultaneous analysis of ciprofloxacin, sulfasalazine and cortisone which are structurally different compounds. The phases herein used do not require long preparation times or special storage conditions. The validated method reported here

Table 5
Intra-day and inter-day precision (RSD%) and trueness (Bias%) of the analytical method obtained from the analysis of QC samples in whole blood, plasma, and urine samples.

Matrix	Analyte	Conc.[a] (μg/mL)	Intra-day			Inter-day		
			Mean back-calculated (μg/mL)	BIAS%	RSD%	Mean back-calculated (μg/mL)	BIAS%	RSD%
Whole blood	Ciprofloxacin	0.8	0.77	−3.8	8.3	0.81	1.12	6.2
	Sulfasalazine		0.82	2.5	8.3	0.78	−2.32	3.3
	Cortisone		0.82	2.4	11.6	0.85	6.39	7.5
	Ciprofloxacin	2.5	2.28	−8.8	3.8	2.45	−1.93	3.6
	Sulfasalazine		2.69	7.5	6.4	2.54	1.47	2.5
	Cortisone		2.54	1.6	6.8	2.53	1.12	3.9
	Ciprofloxacin	8.0	7.92	−1.0	9.5	7.72	−3.47	3.5
	Sulfasalazine		8.81	10.2	7.1	7.77	−2.83	2.6
	Cortisone		8.73	9.2	4.2	8.26	3.19	9.2
Plasma	Ciprofloxacin	0.8	0.76	−5.6	10.1	0.72	−10.5	2.1
	Sulfasalazine		0.83	3.3	7.5	0.79	−1.54	6.9
	Cortisone		0.81	0.9	9.9	0.77	−3.28	11.2
	Ciprofloxacin	2.5	2.42	−3.1	9.2	2.48	−0.66	8.7
	Sulfasalazine		2.47	−1.1	10.9	2.61	4.58	7.3
	Cortisone		2.56	2.2	8.1	2.26	−9.70	5.3
	Ciprofloxacin	8.0	8.10	1.2	7.7	7.87	−1.63	10.5
	Sulfasalazine		7.48	−6.4	5.6	8.21	2.67	6.0
	Cortisone		7.13	−10.9	2.1	7.13	−10.9	1.8
Urine	Ciprofloxacin	0.8	0.79	−1.5	7.3	0.74	−7.63	4.8
	Sulfasalazine		0.79	−1.5	8.1	0.84	4.86	7.2
	Cortisone		0.79	−1.3	7.5	0.81	1.82	8.3
	Ciprofloxacin	2.5	2.54	1.5	7.5	2.49	−0.20	7.0
	Sulfasalazine		2.52	0.9	10.6	2.60	4.07	6.8
	Cortisone		2.34	−4.3	12.3	2.29	−8.41	6.8
	Ciprofloxacin	8.0	8.82	10.2	4.6	8.34	4.19	6.3
	Sulfasalazine		8.32	4.0	8.6	7.93	−0.82	10.2
	Cortisone		7.52	−6.0	7.2	7.19	−10.1	0.8

The data are the mean values of six experiments (n = 6).
[a] Theoretical drug concentration.

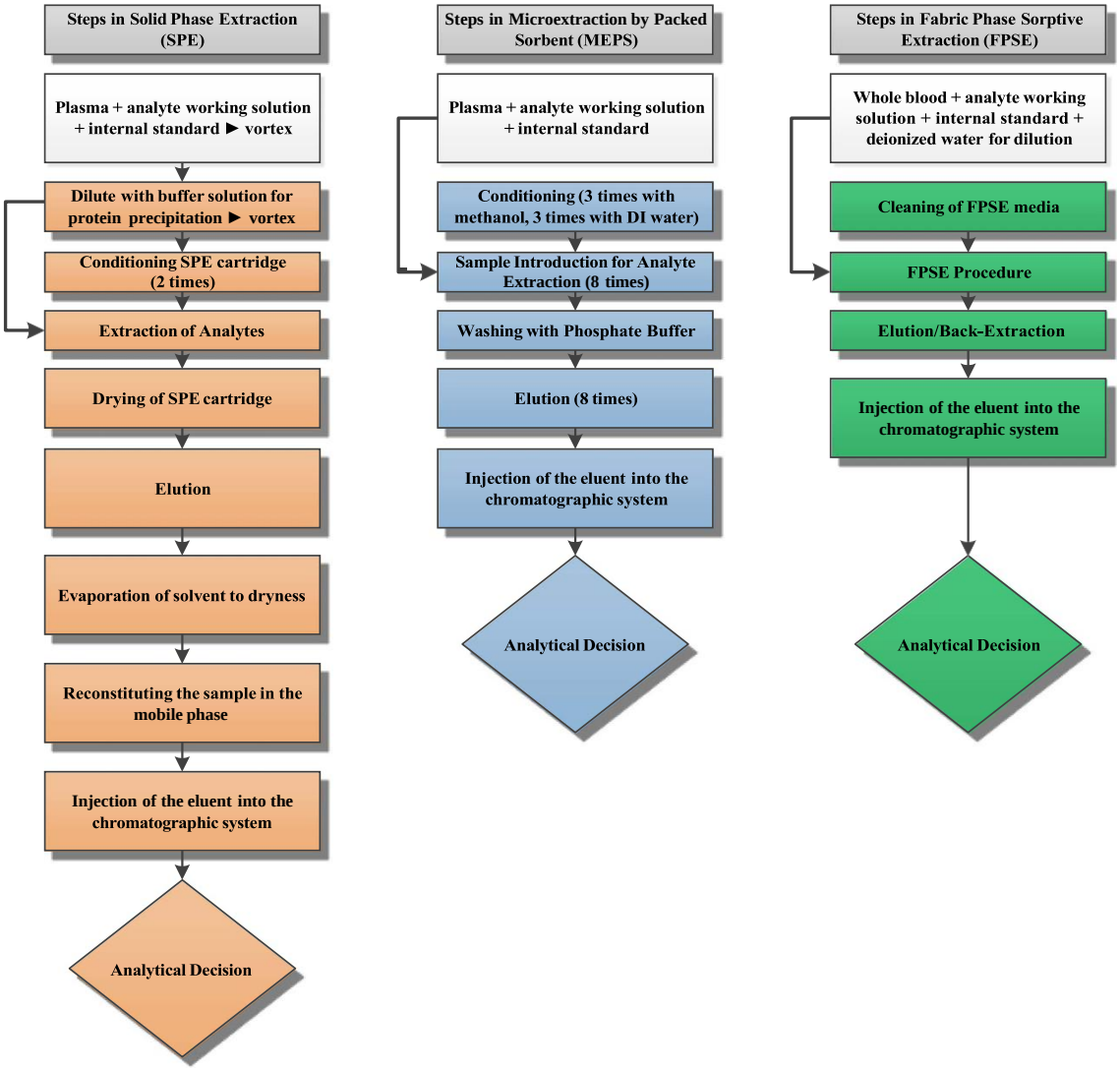


Fig. 3. Schematic representation of the steps involved in SPE, MEPS and FPSE.

requires HPLC-PDA as the chromatographic system, does not warrant qualified and highly trained personnel as in the case when dealing with LC-MS/MS [4,41,42].

A performance summary of different methods have been reported in Table 6.

3.8. Application to clinical whole blood, plasma and urine samples

The performances of analytical method were tested in whole blood, plasma and urine samples collected from IBD patients during their normal clinical therapy protocol. Physiological samples were extracted by FPSE device and quantified using HPLC-PDA according to the vali- dated method reported herein. Table 7 reports the quantitative data obtained after clinical samples analyses. The data revealed that the residues of IBD treatment drugs could be monitored directly from the whole blood. However, more clinical data is needed to establish any potential correlation present in the concentration of the IBD drug re- sidues in whole blood, plasma and urine so that analytical data in one physiological sample would allow to estimate the concentration of drug residues in other sample matrices at a high level of confidence.

4. Conclusion

For the first time, a new fabric phase sorptive extraction-high performance liquid chromatography-photodiode array detection method has been presented for simultaneous monitoring of three IBD drug residues in whole blood, plasma and urine samples. The new ap- proach successfully eliminates protein precipitation, solvent evapora- tion and sample reconstitution from the sample preparation workflow. Important fabric phase sorptive extraction parameters such as suitable sorbent type, FPSE media dimension, sample volume, back-extraction solvent and its volume were optimized. Major HPLC-PDA methodparameters including mobile phase pH, ionic strength of the buffer solution, temperature of the chromatographic column and mobile phase composition were carefully evaluated and optimized during method development. Analytical figures of merit obtained from the validationprocedure showed that the FPSE-HPLC-PDA assay could be applied to detect and quantify ciprofloxacin, sulfasalazine, and cortisone in whole blood, plasma and urine sample matrices with high degree of con- fidence without requiring any expensive chromatographic instrument such as LC-MS or LC-MS/MS. Due to the substantial simplification of the sample preparation using FPSE, the method uncertainty and the overall total analysis time have been considerably reduced. FPSE in- deed opens up a new direction in whole blood analysis with potential future applications in metabolomics disease biomarker discovery, pharmacokinetic, pharmacodynamics, toxicology and related clinical investigations where plasma or serum is still being routinely used as the primary investigational sample matrix as the proxy for whole blood.

Table 6
Comparison between the current FPSE-HPLC-PDA method and other reported methods.

Sample	Analytes	Extraction	Instrument	Extraction (min)	Chromatographic (min)	Dynamic range	Ref.
Human plasma	*Sulfasalazine* Metabolite sulphapyridine 5-Aminosalicylic acid	PP	LC–ESI-MS/MS	6	9	10–10,000 ng/mL	[3]
Human plasma	*Ciprofloxacin*	PP	HPLC–UV/Vis	12	5	0.05–8 ng/mL	[40]
Human plasma	*Ciprofloxacin*	PP	HPLC-FLD	12	8	0.02–4 ng/mL	[6]
Human plasma	Posaconazole Voriconazole Itraconazole Hydroxy-itraconazole Daptomycin *Ciprofloxacin* Oxacillin Levofloxacin Rifampicin Imatinib Raltegravir	LLE	HPLC–MS/MS	10	5	n.d.	[4]
Human urine	Cortisol *Cortisone* Tetrahydro-metabolites	PP	HPLC-MS/MS	12	25	0.1–160 ng/mL 0.1–160 ng/mL 0.2–160 ng/mL	[35]
Human urine	Cortisol *Cortisone* Tetrahydro-cortisol Tetrahydro-cortisone Allo-tetrahydro-cortisol Allo-tetrahydro-cortisone	PP	HPLC-MS/MS	30	13	1–1000 ng/mL 1–1000 ng/mL 1–5000 ng/mL 1–5000 ng/mL 1–1000 ng/mL 1–1000 ng/mL	[36]
Human urine	Cortisol *Cortisone*	Centrifuge + SPE	HPLC–MS/MS	5	20	0.6–150 ng/mL 0.8–200 ng/mL	[37]
Human plasma	Cortisol *Cortisone* Prednisolone Prednisone	LLE	HPLC–MS/MS	45	8.0	1–500 ng/mL 0.5–251 ng/mL 2–1000 ng/mL 0.5–500 ng/mL	[38]
Human urine	Cortisol *Cortisone*	Centrifuge	HPLC-UV/Vis	20	24	10–160 ng/mL.	[8]
Human plasma, urine	Cortisol *Cortisone* Tetrahydro-cortisol Tetrahydro-cortisone Allo-tetrahydro-cortisol Allo-tetrahydro-cortisone	–	HPLC-FLD	–	30	5.0–1000.0 ng/mL 5.0–1000.0 ng/mL 5.0–1000.0 ng/mL 10.0–1000.0 ng/mL 10.0–1000.0 ng/mL 10.0–1000.0 ng/mL	[39]
Human saliva, plasma, blood, urine	Cortisol Cortisone Corticosterone	MEPS	HPLC-DAD	–	20	5–100 ng/mL	[7]
Human whole blood, plasma, urine	Cortisone Ciprofloxacin Sulfasalazine	FPSE	HPLC-PDA	30	20	0.05–10 μg/mL (WB) 0.25–10 μg/mL (Plas) 0.10–10 μg/mL (Uri)	[Current study]

PP = protein precipitation; LLE = liquid/liquid extraction; MEPS = microextraction on packed sorbent.

Table 7
Quantitative analysis of whole blood, plasma and urine samples collected from IBD patients during their normal clinical therapy protocol.

Sample ID	Disease	Other therapy	Age	Sex	Treatment	Last administration before sampling	Matrix	Ciprofloxacin	Sulfasalazine	Cortisone
RS1	Ileo-colic Crohn's disease	Budesonide 9 mg/diet	53	Male	Ciprofloxacin® 500 mg 1 × 2/diet (10 days)	14 h	Whole blood Plasma Urine	1.58 BLQ 15.01	– – –	– – –
RS2	Crohn's disease	Golimumab (s.c.) 50 mg/month	47	Male	Salazopyrin® 500 mg 2 × 2/diet (approx. 4 years)	13 h	Whole blood Plasma Urine	– – –	2.49 4.75 5.83	– – –

BLQ: Below Limit of Quantification (whole blood 0.05 μg/mL; plasma 0.25 μg/mL; urine 0.1 μg/mL).

Acknowledgement

This work was supported by grant MIUR ex 60%, University of Chieti-Pescara “G. d'Annunzio”, Chieti, Italy.

Conflict of interest

The authors report no conflicts of interest.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2018.03.028.

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