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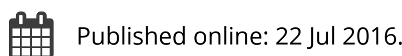

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RESEARCH ARTICLE

Microextraction by packed sorbent and HPLC–PDA quantification of multiple anti-inflammatory drugs and fluoroquinolones in human plasma and urine

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Abstract

We developed and validated an analytical method based on microextraction packed sorbent (MEPS) and high-performance liquid chromatography (HPLC) coupled to photodiode array (PDA) detector to simultaneously quantify multiple nonsteroidal anti-inflammatory drugs (NSAIDs) and fluoroquinolones (FLQs), which may provide as combination several adverse reactions in nephrology and neurology. The linearity range from LOQs (0.1 μg/mL) to 10 μg/mL, and LODs values were 0.03 μg/mL for both NSAIDs and FLQs. The validation was performed according to international guidelines and the accuracy was tested measuring the precision, intermediate precision and trueness. The drugs stability was tested under different storage conditions (+4 °C and −20 °C) and after three different cycles of freezing and thawing. The method can be a suitable tool to simultaneously detect a possible association of drugs in human biological samples and provide several potentialities for clinical applications, bioequivalence studies, pharmacodynamics and toxicodynamics of different pharmaceutical dosage forms showing NSAIDs and FLQs.

Keywords

MEPS–HPLC–PDA, method validation, NSAIDs and fluoroquinolones, plasma and urine, sample preparation

History

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Introduction

NSAIDs is the acronym of nonsteroidal anti-inflammatory drugs, which are used to treat from acute to moderate painful occurrences, and are often ''abused'' in the treatment of an inflammatory pain. The NSAIDs inhibit the phospholipase A2 and cyclooxygenase, thus decreasing the biosynthesis of prostanoids. Actually, there are three cyclooxygenase isoforms: COX-1, COX-2 and COX-3. The first is constitutive of gastrointestinal tract tissues and inhibits the synthesis of prostanoids. The COX-2 is expressed in the kidney, gastrointestinal tract and central nervous system (CNS)[1], and its expression depends on the inflammation process and leads mainly to the production of prostaglandin E2 (PGE2) or other prostaglandins that increase the pathological occurrence[2]. The COX-3, which has an unknown role in inflammatory processes, seems to be the main target of paracetamol drug. The anti-inflammatory effect of NSAIDs depends on the inhibition of COX-2 isoform, while the inhibition of COX-1 can provide several side effects, particularly at the gastrointestinal tract[2]. The COX-1 mediates the production of vasodilator induced by prostaglandin (PG) that preserves the physiological function of kidneys, for example, renal plasma flow and glomerular filtration. The abuse of anti-inflammatory drugs suppresses this protective function, thus producing a renal ischemia[1–4]. The inhibition of COXs activity promotes the metabolism of arachidonic acid, thus increasing the release of leukotrienes, which extend the inflammatory response. Additionally, some COX inhibitors can act also on other biological targets, such as celecoxib that shows inhibition activity on carbonic anhydrase[5].

Fluoroquinolones (FLQs) are synthetic antibiotics originated from quinolone and its derivatives[6]. FLQs show a 4-quinolone core and 3- and 4-carboxy carbonyl pending chains, which mediated the antibacterial activity. The presence of substituents at C-7 improves the pharmacokinetic, intracellular stability and increases the antimicrobial activity versus Gram-positive bacteria[7,8].

The blockbuster of NSAIDs, their abuse in therapy and low costs can promote several side effects, which may be related to the intake with other drugs. Conversely, the synergistic effect between FLQs and NSAIDs can offer some therapeutic benefits *in vivo* as previously reported[9]. Unfortunately, the association between FLQs and NSAIDs can provide several side effects. In particular,

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the ciprofloxacin induced a nephropathy in young patients without any specific renal diseases. The biopsies of tissue showed an acute tubular necrosis with intratubular needle-like crystals[10]. Furthermore, NSAIDs and FLQs are suspected to induce neuro-psychiatric adverse reactions in patients under therapeutic treatment[11]. These drawbacks need a click and easy method to quantify NSAIDs and FLQs in biological fluids for multiple drugs therapy.

The aim of this study is to develop and validate an analytical method suitable to separate and quantify NSAIDs and FLQs in biological samples, for example, plasma and urine, by a single chromatographic analysis. The analysis of sample was carried out using HPLC–PDA apparatus, which is easily used for routinely analyses in several chemical and bioanalytical laboratories, and the method was validated according to the international guidelines and requirements[12–14].

The MEPS procedure was used to optimize the extraction and recovery of samples and the drawbacks related to conventional methods of sample preparation.

We previously demonstrated that HPLC–PDA is a suitable analytical method to separate and quantify different drugs classes at the same time from biological samples[15–18]; however, no MEPS–HPLC–PDA method is actually available to separate and quantify these two different classes of drugs. In this paper, the MEPS–HPLC–PDA was used to analyze simultaneously seven different NSAIDs and four FLQs (Figure 1), which show different physical–chemical properties and backbone structures. The MEPS–HPLC–PDA was developed in human plasma and urine. The drugs peaks are resolved and did not show any interferences and overlapping with biological samples. The resulting LOD, LOQ and ULOQ values demonstrated that NSAIDs and FLQs were validated in human plasma and urine according to the international guidelines and requirements.

Methods

Chemical and reagents

The anti-inflammatory drugs (furprofen, indoprofen, ketoprofen, fenbufen, flurbiprofen, indomethacin, ibuprofen), fluoroquinolones (ciprofloxacin, levofloxacin, moxifloxacin, ulifloxacin), internal standards or IS (enrofloxacin and sarafloxacin) and ammonium acetate were purchased from Sigma–Aldrich (St. Louis, MO).

Methanol, acetonitrile, triethylamine and trichloroacetic acid were purchased from Carlo Erba Reagents (Milan, Italy) and were of analytical grade. The double-distilled water was obtained by Millipore Milli-Q Plus water treatment system (Millipore Bedford Corp, Bedford, MA). Sodium hydrogen phosphate ($\geq 99\%$), sodium dihydrogen phosphate (99%) and *o*-phosphoric acid were of analytical grade and were purchased from Sigma-Aldrich (Milan, Italy). The MEPS device and replacement needle with C_{18} stationary phase were purchased from SGE Analytical Science (Trajan Scientific Europe Ltd, Crownhill, Milton Keynes, UK).

Oki® (80 mg) and Momentact® (400 mg) were purchased in local pharmacy and were from Dompé Farmaceutici S.p.A (Milan, Italy) and Gruppo Angelini (Ancona, Italy), respectively.

Sampling and storage of samples from human volunteers

The plasma and urine were sampled from two adult healthy volunteers 5 h after oral administration of NSAIDs or FLQs. The biological samples were collected by venous sampling and stored at $-20\,^{\circ}C$ before the analysis. The volunteers were informed about the experimental procedures and the nature of the study and provided a written consent before the experiments. The volunteers were healthy and were not under medications before the

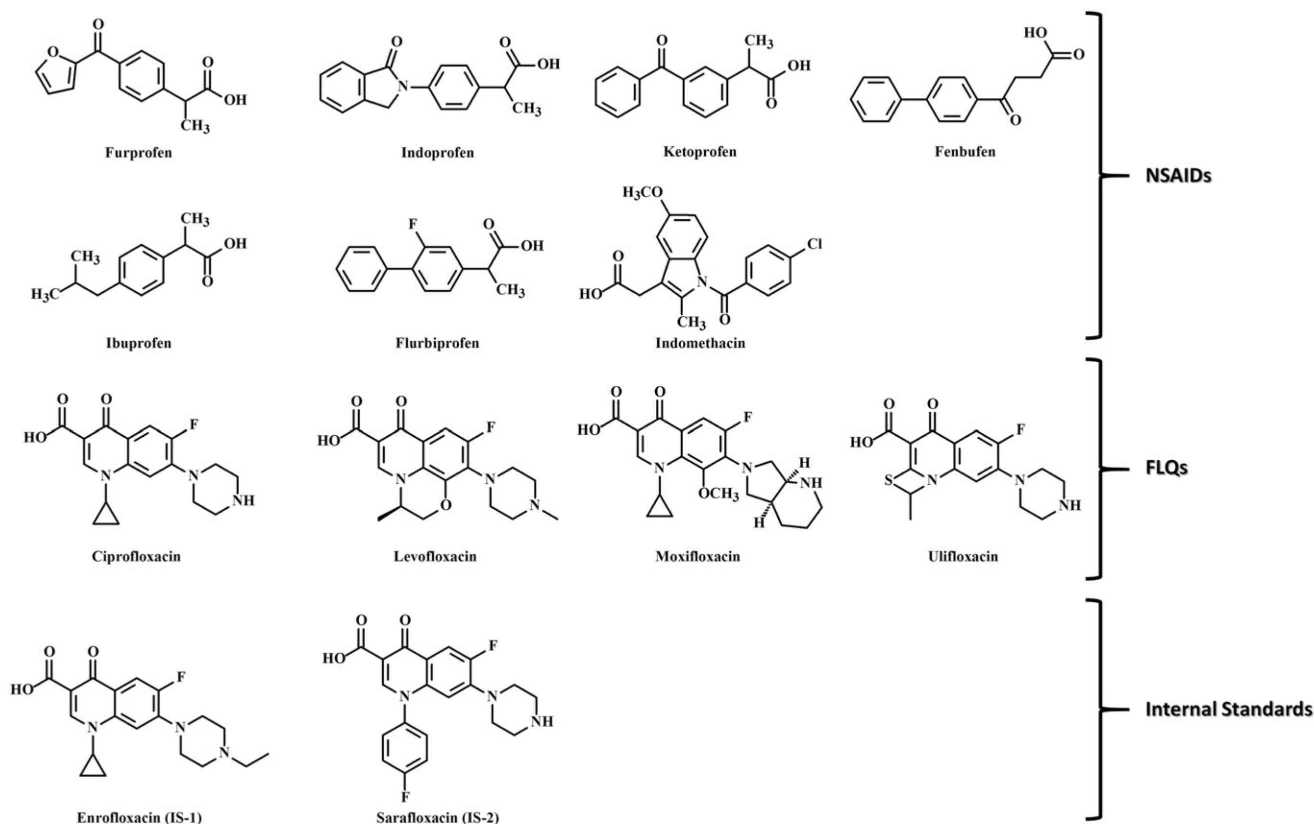

Figure 1. Chemical structures of NSAIDs, FLQs and internal standards.

experiments. We analyzed using a new method compounds and samples previously reported[17].

Biological sample preparation (plasma and urine)

Human plasma and urine (180 µL) were added with working solutions (10 µL) of drugs and working solution (10 µL) of internal standards (2 µg/mL for enrofloxacin and 10 µg/mL for sarafloxacin, respectively). The resulting samples were vortex-mixed for 1 min, thus modifying of 10% its final composition (5% in biological samples).

A preliminary cleaning of samples was carried out by adding trichloroacetic acid (100 µL); the resulting sample was further centrifuged at 12 000 × *g* for 5 min, thus removing the denatured proteins, hydrolyzing protein residue-binding drugs and decreasing the sample density to facilitate its passage through the MEPS stationary phase as previously reported[19]. A MEPS syringe apparatus of 250 µL nominal volume with a sorbent stationary phase (C_{18}) was used for the extraction of different samples.

The preliminary cleaning of MEPS provided a performance of extraction over 90 samples before replacing needle and cartridge. Furthermore, the needle maintain its efficiency and accuracy over time during the analysis. Multiple suctions (in and/or out) of nominal volume of syringe was performed at each step to increase the overall efficacy of procedure.

The MEPS apparatus was optimized as below reported: sorbent conditioning (150 µL of methanol for 3-fold); sorbent equilibration (150 µL of ammonium acetate buffer, 50 mM, pH 2.5 for 3-fold); sample loading (100 µL of human plasma or urine for 8-fold); washing step (150 µL ammonium acetate buffer: methanol (95:5, *v:v*)); elution of samples (25 µL of methanol for 8-fold and then directly injected into the HPLC–PDA apparatus).

HPLC apparatus and chromatographic condition

The analyses were carried out by using a HPLC pump system Waters mod. 600 connected to photodiode array detector 2996 (PDA). A Discovery C_8 packed column (4.6 × 250 mm, 5 µm particle size, Supelco, Sigma-Aldrich, Milan, Italy) was used to separate analytes. The analyses were carried out at 25 °C (±1 °C) using a jetstream2 Plus column oven. The mobile phase was directly on-line degassed using Biotech 4CH DEGASI Compact (Onsala, Sweden) and was made from a binary solvent system (phosphate buffer pH 2.5, 30 mM, 1% (*v:v*) TEA, and AcN 1% (*v:v*) TEA) at a flow rate of 1 mL/min. The analyses were optimized using a gradient elution as reported in Table 1.

The sample (20 µL) was directly injected into the HPLC–PDA instrument by MEPS syringe, while the Empower v(0).2 Software (Water Spa, Milford, MA) was used for the acquisition and analysis of data.

Table 1. Chromatographic gradient elution.

Time (min)	% A*	% B†
0	86	14
9	86	14
15	50	50
30	50	50
30	86	14
40	86	14

*Phosphate buffer, pH 2.5, 30 mM, 1% *v/v* TEA.
†AcN, 1% *v/v* TEA; temperature 25 °C ± 1 °C.

Stock solution, calibration and quality control of samples

The stock solutions were made by dissolving the drugs in methanol at 1 mg/mL. The working solutions were obtained by diluting the resulting stock solution in methanol. The different working solutions were in the following range: 2, 5, 10, 16, 20, 40, 80, 100, 160 and 200 µg/mL. The FLQs enrofloxacin and sarafloxacin, approved for veterinary treatment, were used as internal standards. Two different working solutions of 40 µg/mL (enrofloxacin), and 200 µg/mL (sarafloxacin) were diluted in methanol to make the final concentration of internal standards.

Seven calibration points and three quality controls (QCs) for each biological sample were performed as previously reported (biological sample preparation), and the resulting samples were injected into the HPLC–PDA apparatus using MEPS device.

Method validation

The analytical method was validated according to the International Guidelines and requirements[12–14].

The LOD/LOQ, linearity, selectivity, accuracy (intraday and interday) and stability were tested for various drugs in human plasma and urine. The accuracy (precision and trueness) of samples were tested at three QCs concentration levels and the BIAS% and RSD% of resulting data was finally calculated. The calibration curves were obtained by using seven different concentration levels spiked in fresh plasma or urine. The chromatograms of various drugs were detected at different wavelengths (as reported in Supplementary material section S.1) in order to obtain the highest signal/noise ratio.

The concentrations of NSAIDs and FLQs were expressed as the ratio between Analyte and Internal Standard area. The selectivity of the method was tested by comparing the blank and fortified (plasma or urine) samples at the LOQ concentrations of various drugs.

Results and discussion

Optimization of MEPS extraction procedure

Several parameters affect the development of analytical methods, which perform the simultaneous analysis of multiple drugs. To solve samples and separate different drugs from biological samples, the extraction procedure should be optimized in terms of cleaning, number of processes, solvent volume and area of peaks. In this attempt, the preparation of samples at the early stage of analysis plays a crucial role for the efficiency of analysis[20].

Biological samples, for example, plasma and urine, contain proteins and different elements, which can interfere with analysis. These biological compounds are usually removed before the HPLC analysis by adding trifluoroacetic acid[21], which denature proteins without affecting the physicochemical features and backbone structure of drugs. The preparation of samples needed different steps, which dilute and stress mechanically[20,22] the compounds before their loading in MEPS apparatus[20].

Furthermore, the extraction procedure by MEPS apparatus stressed samples during their optimization because they undergo different processes such as the conditioning, equilibration, loading, washing and elution. In particular, the pressure variation during the loading and injection of samples by MEPS apparatus increases the interaction between compounds and adsorbent phase, thus critically affecting the efficiency of final extraction[19]. The impact of pressure for the optimization of extraction becomes critically for manual procedures[19].

The ammonium acetate buffer at pH of 2.5 forms ionic derivatives for acid species and decreases their solubility in the sample solution. These physicochemical properties of buffer

provide a better transfer of acid drugs into the organic phase[23], thus improving the performance of extraction.

The repeated charging and discharging of compounds through the same microvials affect the recovery of samples and their peak area, thus increasing the number of extractions from 1 to 8 at the same volume (100 μL)[17,19].

The washing procedure further affects the MEPS performance; in fact, the ratio between aqueous and organic solvents, dissolving samples, can decrease the drug content during the MEPS extraction due to the retention effect and distribution of drugs in aqueous or organic phase[19]. This effect related to the physicochemical features of compounds can provide a leakage of drugs before the HPLC injection[17]. In this attempt, we optimized the washing procedure by changing the composition of solvent mixture and its final ratio. Firstly, we only used aqueous ammonium acetate buffer, but multiple peaks of compounds and several interferences were carried out by analyzing the chromatograms of biological samples.

Secondly, we used a mixture of ammonium acetate buffer and methanol (95:5, *v:v*), thus permitting a better resolution of HPLC chromatograms in biological samples without any multiple and interference peaks. Furthermore, we used a single washing of samples, thus decreasing the spread of samples during the MEPS extraction and simplifying the procedure.

The elution of compounds dispersed in biological samples depends on physicochemical features of organic solvent, which is used to make the extraction. Multiple washings (4–5 cycles) provide a carryover below 2% and the maximum volume of extraction of compounds from biological samples as previously reported[17,19,24]. For this reason, the elution of compounds from human and urine samples was set up according to literature to have the maximum volume of extraction and improve the performance of MEPS procedure.

HPLC separation and development of method

The HPLC method was developed and optimized by using different chromatographic columns and mobile phases. The mobile phases were also modified using different organic solvents and ratios with aqueous buffers. We previously demonstrated that phosphate buffer (30 mM, pH 2.5) and AcN mobile phase (58:42, *v:v*) at 40 °C using a C_{18} column provided a suitable resolution of chromatograms of NSAIDs with separate peaks at different retention times[17]. Furthermore, we demonstrated that the FLQs (e.g. ciprofloxacin, levofloxacin and enrofloxacin) can be separated in isocratic condition by using a phosphate buffer (30 mM, pH 2.5, 1% (*v:v*) TEA) and AcN (1% (*v:v*) TEA) mobile phase (86:14, *v:v*) at 25 °C using a C_8 column[15].

According to data previously reported[15,17], we optimized method by using isocratic instead of gradient elution, and try to remove TEA from aqueous buffer and organic solvent. These changes can provide a standard HPLC method, which can easily move from different apparatus, without any shift of chromatographic peaks and retention times of compounds. The removing of TEA can further enhance the safety of analytical method. In this attempt, we tested different mobile phases, which are obtained by combining phosphate buffer (30 mM, pH 2.5), AcN, and methanol at different volume ratio. Two different columns, particularly a C_{18} and C_8, setting at 25 °C and in isocratic condition, were used as stationary phase.

Firstly, we performed the analysis by using phosphate buffer (30 mM, pH 2.5):AcN:methanol at the ratio 80:10:10, 15:5:80 and 80:15:5 (*v:v:v*). Unfortunately, we obtained several peaks for NSAIDs, which are overlapped in range of analysis from 4 to 12 min. The adding of TEA to mobile phases at different ratio (0.1, 0.3 and 0.5%, *v:v*) did not solve the chromatographic peaks of NSAIDs and their shape and symmetry was not suitable to quantify simultaneously multiple drugs. Furthermore, the isocratic elution were not suitable to solve and separate FLQs. At different ionic strength of phosphate buffer (10–30 mM), we showed that an increase of buffer concentration improved the peak shape and retention time of different compounds compared to mobile phase herein reported. For these reasons, we run chromatographic analysis in gradient condition and using different solvents ratio.

Unfortunately, many compounds were coeluted with mobile phase using gradient configuration and the resulting peaks were overlapped during the analysis. To overcome these drawbacks, we tested C_{18} and C_8 columns at two different temperatures, for example, at 30 °C and 40 °C. The increase of temperature did not avoid the overlapping of chromatographic peaks and affected negatively the retention of different compounds in the stationary phase, thus changing the distribution of drugs between mobile phase and columns. Finally, we separated the NSAIDs and FLQs, after MEPS extraction from biological samples, by using a gradient condition. The composition of mobile phase was optimized by combining the single gradient procedures, which allow solving NSAIDs and FLQs separately.

In particular, the mobile phase was made starting from phosphate buffer (30 mM, pH 2.5):AcN (86:14, *v:v*) for FLQs[15], and then moving to phosphate buffer (30 mM, pH 2.5):AcN (60:40, *v:v*) for NSAIDs[17]. The elution of samples using the resulting gradient provided an overall runtime of 40 min; the mobile phase was eluted at 86:14 (*v:v*) by 15 min and then moved to 50:50 (*v:v*) by 30 min. A re-equilibration run of 10 min was carried out to have the original conditions of the analysis. The chromatogram showed a slight increase in baseline when the mobile phase changes its composition during the analysis; however, this modification does not interfere with LOD, LOQ and linearity of samples. A better resolution of these parameters could be performed by using mass spectrometry (MS) detector; however, the use of MS detector will require several set up and validations, which are time consuming and expensive for an easy and quick validated method.

Method validation

The validation of linearity, accuracy (precision and trueness), sensitivity, selectivity and recovery were performed according to International Guidelines[12–14].

Linearity, LOQ and accuracy

The calibration curves were obtained by plotting the corrected area of samples (ratio between the area of different compounds and IS) versus the nominal concentration of each standard solution. Different levels of unknown samples and standard solution were carried out during the analysis.

The linearity of the method was tested by measuring the intercept, slope, determination coefficient and variations of samples in the range from 0.1 to 10 μg/mL of different compounds. The over range was tested using a weighted linear least-squares linear-regression determination coefficient (r^2) ≥ 0.9720 and a weighting factor of ($1/x^2$), according to the method validation requirement of International Guidelines[13].

The LOQ values are 0.10 μg/mL for different compounds; while the LODs of the method was set at 0.03 μg/mL according to the signal-to-noise ratio.

The fortified urine samples showed a better signal-to-noise ratio then plasma samples. The LOD and LOQ of urine samples cannot be used as reference to set up the minimum signal-to-noise

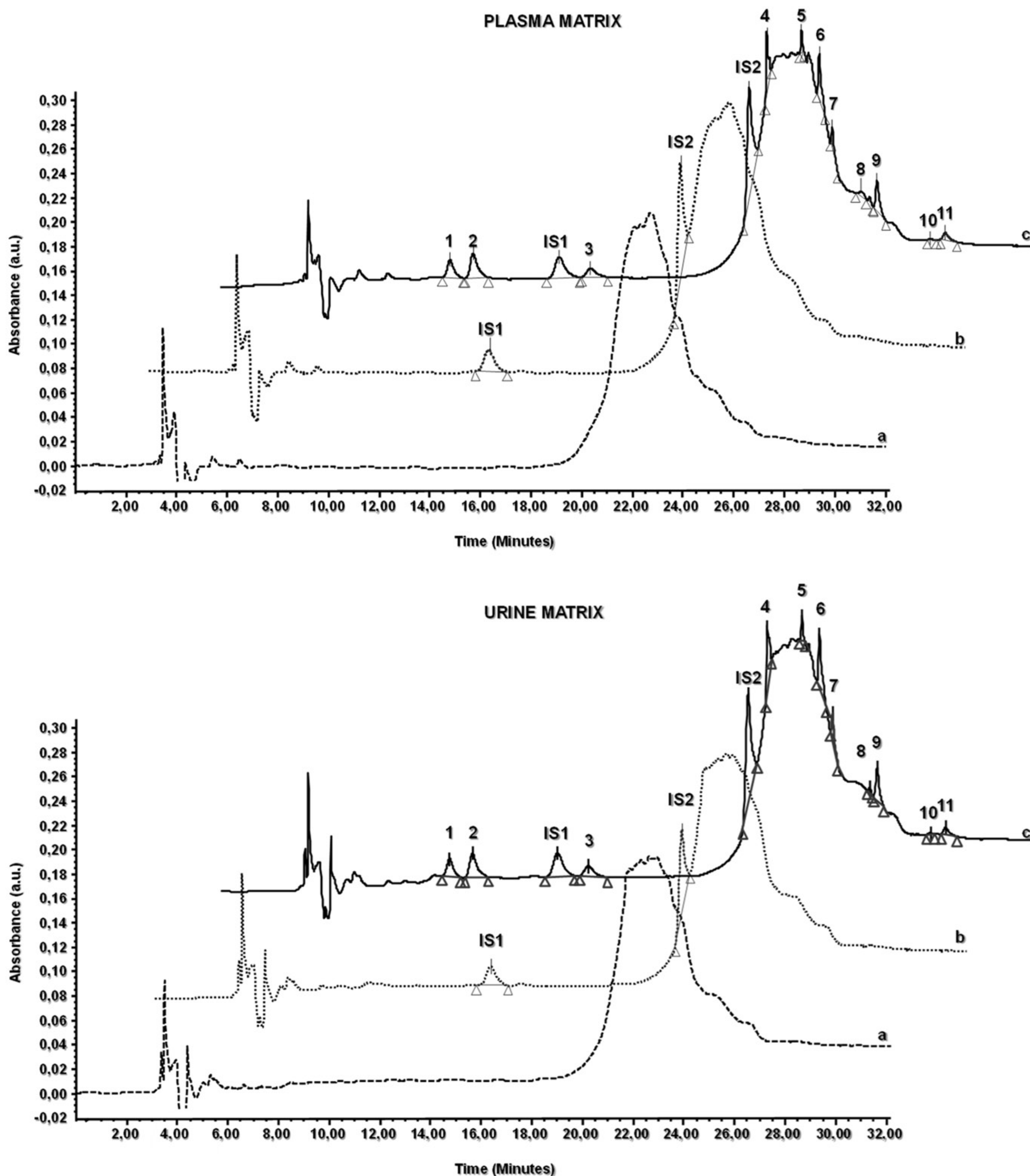

Figure 2. Chromatograms of different samples after MEPS extraction. Top side, plasma MEPS–HPLC–PDA chromatogram: (a) double blank, (b) blank spiked with internal standards and (c) blank spiked with internal standards and 1 μg/mL of all analytes. Bottom side, urine MEPS–HPLC–PDA chromatogram: (a) double blank, (b) blank spiked with internal standards and (c) blank spiked with internal standards and 1 μg/mL of all analytes. About 20 μL of samples injected at 285 nm. Key legend: levofloxacin (1), ciprofloxacin (2), enrofloxacin (IS1), ulifloxacin (3), sarafloxacin (IS2), moxifloxacin (4), furprofen (5), indoprofen (6), ketoprofen (7), fenbufen (8), flurbiprofen (9), indomethacin (10) and ibuprofen (11).

ratio because its precision and bias% values were not agree with the ICH guidelines, which showed a precision below 20% and the trueness in the range from 80% to 120%.

The QC samples were analyzed at different days and after MEPS extraction through different needles. This procedure allowed testing the validation between assay precision (intermediate precision) and trueness, and the variability among different needles of different biological samples (as reported in Supplementary material section S.2).

The selectivity of the method was evaluated by analyzing eight different plasma and urine samples (Figure 2) according to the ICH guidelines[12]. The analysis of blanks showed that the LOQ areas were not over 20% and 5% at retention times of different samples and IS.

Table 2. Quantitative analysis of NSAIDs contained in commercial medications after the oral administration in human volunteers.

	Volume (mL)	Time (hours)	Concentration (μg/mL)	Total concentration (mg)	Concentration (μg/mL)	Total Concentration (mg)
Plasma						
Oki® 80 mg	4400*	5	4.02	17.7	3.95†	17.4†
Momentact® 400 mg	4400*	5	9.56	42.1	8.73†	38.4†
Urine						
Oki® 80 mg	400*	5	0.93	0.37	0.89†	0.36†
Momentact® 400 mg	200*	5	1.19	0.24	1.31†	0.26†

*These values were obtained from literature[27].
†These values were previously published in literature[17].
The other data are experimental results, which were compared to data previously published to control the performance of the HPLC–PDA and MEPS extraction.

The optimized HPLC–PDA procedure demonstrated that the retention times of various compounds, extracted from biological samples by MEPS apparatus, were similar to the retention times of real samples and no interfering peaks were obtained during the analysis (Figure 2). Furthermore, a change in compounds and IS concentrations were not observed during the analysis and after storage conditions as well as any variations of their chromatograms due to a potential chemical and/or photochemical degradation.

Parallelism test

The parallelism test of different samples was carried out by comparing compounds (high concentrations) diluted (1:100, *v:v*) in plasma and urine with the pooled relative biological samples, which were used to make standards and QC analysis. In fact, samples (high concentrations) diluted in plasma and/or urine over the upper values of calibration curve (200 μg/mL) can be measured with the same accuracy of compounds, which have a concentration within the linear range of analysis.

Application of the method to plasma and urine samples

The analytical method was also validated using ketoprofen and ibuprofen drugs of commercial medications orally administered in human volunteers (Oki® 80 mg or Momentact® 400 mg) and plasma or urine were collected 5 h after injection.

The analyses of commercial medications (Oki® and Momentact®) and chromatograms of commercial medications collected from plasma and urine of human volunteers were comparable to data of NSAIDs used to develop and validate the method (Table 2). Results agreed data previously reported[25,26]. Furthermore, the resulting method shows similar parameters compared to the analysis of NSAIDs previously reported[17].

Conclusions

The MEPS extraction combined with HPLC–PDA allows to separate and quantify 11 drugs (7 NSAIDs and 4 FLQs). The analytical method increased the recovery of samples and decreased the matrix effect, thus improving the overall procedure of extraction and analysis, confirming the recent reported MEPS potential[28]. Several tests were performed to optimize and set up the best conditions of mobile phase, type of columns and temperature of analysis for chromatographic separation. The instrument set up was firstly optimized for FLQs and moved to NSAIDs, thus finally obtaining a suitable analytical method to quantify simultaneously 11 drugs extracted from biological samples (plasma and urine). The resulting procedure is accurate, selective and precise in the range of analysis and can be used to monitor the combination between NSAIDs and FLQs in clinical trials, pharmacological and toxicological studies. The analysis of commercial medications oral administered in human volunteers demonstrated that the analytical procedure can be used for the quantitative analysis of drugs in real samples.

The combination of MEPS extraction and HPLC–PDA apparatus allowed to analyze multiple drugs with different physicochemical features in easy and quick mode without the use of complex extraction procedures and instrumental apparatus.

Declaration of interest

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The authors declare no conflict of interests.

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