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HILIC-DAD Method for Simultaneous Determination of Acid and Basic Drugs: Application to the Quantitation of Ibuprofen, Atenolol, and Salbutamol in Urine After Solid-Phase Extraction

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Abstract: A simple method has been developed for the simultaneous analysis of ibuprofen (acid drug), and salbutamol and atenolol (basic drugs) in urine samples at concentrations of 0.40 μg·mL^{-1}. Simultaneous chromatographic separation has been possible using hydrophilic interaction liquid chromatography (Kinetex HILIC® column (2.1 mm × 150 mm, 2.6 μm particle size diameter and 100 Å pore size) combined with gradient elution by employing a mixture of acetonitrile–acetate buffer 5 mM at pH 6 (from 95:5 to 75:25 (*v*/*v*)) as the mobile phase. Detection was performed at 227 and 275 nm. The simultaneous preconcentration and cleaning of the sample has been possible by solid-phase extraction using the HLB ExtraBond® polymeric-type sorbent (which is a pyrrolidone-modified divinylbenzene polystyrene type). It has provided recoveries between (63 ± 9)% for salbutamol, (74 ± 8)% for ibuprofen, and (96 ± 9)% for atenolol in 10 mL of synthetic urine containing 4.0 μg of each of the drugs analyzed. The detection limits were 0.025 μg·mL^{-1} for ibuprofen, μg·mL^{-1} for salbutamol, and 0.007 μg·mL^{-1} for atenolol. The detection limits obtained allow the evaluation of the free forms of ibuprofen, atenolol, and salbutamol at the excreted concentration levels at the therapeutic doses usually administered. The coefficients of variation between days were in the range 4.5–10.9%.

Keywords: HILIC-DAD separation; acidic and basic drugs; SPE sorbents; urine

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1. Introduction

The simultaneous analysis of acidic and basic drugs in samples of biological origin such as serum, plasma, or urine is a difficult challenge to address in chromatographic analysis since both the sample preparation conditions and the chromatographic separation usually use different methodologies, given the differential characteristics of the analytes to be studied. In chromatographic techniques such as liquid chromatography (HPLC), it is essential that we select the stationary phase and the nature of the mobile phase to obtain an adequate chromatographic separation of the analytes present in the sample. On the other hand, the presence of endogenous matter existing in plasma, urine, or serum samples can make it difficult to determine many analytes by causing overlaps of chromatographic peaks or decreasing the sensitivity of detection. This matrix effect requires a sample pre-treatment step. Among the available treatments, solid-phase extraction (SPE) is one of the most used ones. It employs different sorbents that can improve both the selectivity and sensitivity of further chromatographic analysis. However, it is essential that we select the appropriate nature of the sorbent and the elution conditions to achieve the optimal recovery of the analytes to be determined.

In this study, three drugs have been chosen, atenolol, ibuprofen, and salbutamol (Figure 1), which are prescribed for various diseases and can be consumed simultaneously [1].

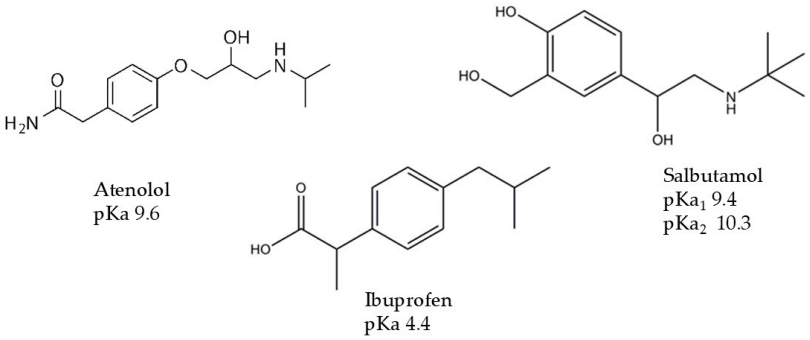

Figure 1. Molecule structures and p*Ka* of atenolol, ibuprofen, and salbutamol.

Ibuprofen is considered an acidic drug, while atenolol and salbutamol are in the group of basic drugs. Atenolol is a β-blocker used in the control of blood pressure [1,2]. It is highly polar and its determination in complex samples is mainly performed by HPLC [3–7], since, for the determination by gas chromatography, a prior derivatization is necessary, which is usually incomplete [3]. Most separations are performed with silica-bonded phases such as C18 and C8 [3–6], by employing mobile phases with different ratios of acetonitrile or methanol and aqueous solutions acidified with formic acid [3], or phosphoric acid [6]. Salbutamol is a short-acting β_2 adrenergic receptor agonist frequently used to treat asthma [8]. As in the case of atenolol, salbutamol can be determined by gas chromatography with mass spectrometry after a derivatization process [3], although the most commonly used separation technique for its analysis in complex samples is HPLC coupled to various detectors [3–5]. The stationary phases and mobile phases used for the salbutamol determination are similar to those used for atenolol [3–5,9]. For example, for the separation of salbutamol and atenolol enantiomers, a heart-cut two-dimensional HPLC technique has been employed. Polar stationary phases based on hydrophilic interaction chromatography (HILIC) were used for the first dimension and organic solvents such as mixtures of acetonitrile and methanol with ammonium acetate buffer at pH 6 were also employed as mobile phases [10]. Regarding ibuprofen, it is one of the most consumed anti-inflammatory substances in Spain [11]. Its determination in complex samples makes it necessary to use separation techniques. In the case of gas chromatography, a derivatization step is required [3], so HPLC is usually the technique of choice. The most commonly used stationary phases are reversed phases such as silica C8 and C18 [3,5,9,12,13], and mobile phases composed of acetonitrile or methanol and aqueous buffer solutions containing formic acid [3,12], trifluoroacetic acid [13], or triethylamine at pH 8 [3,9]. Since ibuprofen is a very polar substance, HILIC separation mechanisms have also been used [14,15] by employing mobile phases with a high acetonitrile content and content of ammonium acetate buffer at pH 6.8. In water samples, the three compounds have been determined by mass spectrometry using a previous separation in a Luna C8 column but using two different mobile phases. In the case of the separation of atenolol and salbutamol, an elution gradient was used with a mixture of acetonitrile and an aqueous solution of 0.1% (*v*/*v*) formic acid, while, for ibuprofen, the elution gradient consisted of an acetonitrile–triethylamine mixture (pH 8) [3]. They have also been determined in meat samples [4] and fluvial biofilm [5] but using different mobile phases for basic and acidic drugs.

For the detection of atenolol, salbutamol, and ibuprofen, UV detectors [3,6,10,12,13], fluorescence detectors [3], the prior derivatization of the analytes, or mass spectrometry (MS) detectors [3–5,9,14,15] have been coupled to HPLC systems. The ionization mode

used in MS is electrospray in the negative ion mode for atenolol and salbutamol, and in positive ion mode for ibuprofen [4,5,9,14,15].

On the other hand, sample preparation is a critical step in the analysis of samples of biological origin, and it may include the cleaning and/or preconcentration of the analytes to achieve quantifiable levels by the selected analysis method. The developed methods include dilution [12,14,15], liquid–liquid extraction (LLE) [6], membrane extraction [16], and SPE [4,5,10,13]. Under ideal conditions, SPE should allow the preconcentration of all the analytes under study and eliminate all interfering substances present in the matrix. However, it is not an easy task when the physicochemical properties of the target analytes are very different. Thus, in the case of atenolol, salbutamol, and ibuprofen, their acid–base behavior is quite different. Among the materials available in SPE, reversed-phase and polymeric sorbents [3,13], suitable for very polar compounds, polymeric materials used as universal sorbents in the hydrophilic–lipophilic balance (HLB) [3,5], mixed-mode SPE cation exchange [3,9,10,13], or mesoporous silica modified with octadecyl silane [4] have been frequently employed.

Another aspect to be considered in the analysis of drugs' biological samples are the possible modification processes and the excretion mechanisms of both unaltered compounds and their possible metabolites. Regarding atenolol, when it is administered orally, the absorption is rapid, but incomplete, since only 10% of the amount absorbed is metabolized. In addition, its majority route of excretion is through the urine and with hardly any transformation [17]. When healthy individuals are treated with doses of 200 $mg \cdot day^{-1}$ for eight days, a half-life ($t_{1/2}$) of between 6 and 8 h is observed and complete elimination of the drug occurs after a period of 48 h. Both the $t_{1/2}$ and the elimination time of the drug are increased when there is kidney damage, and, the longer this is, the more damaged the kidneys are [17]. As for salbutamol, when administered orally, it is well-absorbed through the gastrointestinal tract, and it is excreted in the urine as a mixture of free salbutamol (between 24–33% of the oral dose supplied) and its conjugated metabolites. In the case of inhaled doses, salbutamol is hardly metabolized in the lungs and the amount excreted in the urine depends on the amount inhaled [18]. Trace amounts of salbutamol glucuronide have been observed if salbutamol has been inhaled [19]. In the case of subjects inhaling 0.2 mg of salbutamol four times, the concentration of salbutamol in urine 4 h after inhalation ranged from 8 to 1029 $\mu g \cdot L^{-1}$ for non-asthmatic individuals and was in the order of 1000 $\mu g \cdot L^{-1}$ for asthmatics; in all cases, the concentration of salbutamol glucuronide was less than 2 $\mu g \cdot L^{-1}$. In the case of the oral ingestion of 8 mg of salbutamol, the amounts of salbutamol found in urine range from 1000 to 9320 $\mu g \cdot L^{-1}$ after an analysis at 4 and 12 h after administration and the highest values of salbutamol glucuronide were 63 $\mu g \cdot L^{-1}$; the highest values were observed in asthmatic individuals [19]. Ibuprofen is usually administered as a racemic orally and needs to be administered frequently to maintain therapeutic plasma concentrations in its action as an analgesic, antipyretic, or anti-inflammatory (10–50 $\mu g \cdot L^{-1}$) [20]. Ibuprofen is also excreted unchanged in the urine and as metabolites, the main ones being carboxyibuprofen and 2-hydroxyibuprofen [20]. The metabolism of ibuprofen in humans is due to the reaction with glucuronic acid that is enantioselective with the enantiomer (*S*)-ibuprofen [21]. After the administration of 400 mg of the racemic mixture to healthy volunteers, it is observed that $t_{1/2}$ for the (*S*)-form is 2.3 h, while, for the R form, it is 1.4 h. After 24 h from administration, it was found that 11% of the administered dose was as free ibuprofen, 23% as 2-hydroxyibuprofen, and 40% as carboxyibuprofen [21].

Consequently, in this paper, a novel HPLC method with UV detection was developed for the simultaneous determination of ibuprofen, salbutamol, and atenolol in urine samples at the concentrations usually found after the administration of therapeutic doses. Separation was possible using a Kinetex HILIC® column combined with the gradient elution mode

by employing a mixture of acetonitrile–acetate buffer 5 mM at pH 6 (from 95:5 to 75:25 (v/v)) as the mobile phase. Detection was performed at 227 and 275 nm. A cleaning and preconcentration step by SPE were performed to remove endogenous substances interfering with the identification and quantification of drugs. Additionally, different SPE sorbents, such as the DSC-C18 reversed-phase type, MCX hybrid type (that combine cation exchange and reversed-phase mechanisms), and HLB ExtraBond® polymeric type (which is a pyrrolidone-modified divinylbenzene polystyrene type), were evaluated by performing recovery studies.

2. Materials and Methods

2.1. Chemicals, Reagents, and Solutions

All reagents and solvents were of analytical grade, and purified water from a Milli-Q system (Millipore, Bedford, MA, USA), which was used in all experimental procedures. Pure ammonium acetate, glacial acetic acid (99.8%), ammonia (32%), ammonium hydroxide (30% w/w), hydrochloric acid (37%), supragradient methanol (MeOH), and acetonitrile (ACN) for HPLC were purchased from Scharlab (Barcelona, Spain). Formic acid (98%) was supplied by Merck (Darmstadt, Germany) and phosphoric acid (85%) was obtained from Panreac (Barcelona, Spain). Pure ibuprofen, salbutamol, and atenolol (99%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Chemistry Control Liquichek urine was acquired from Bio-Rad (Hercules, CA, USA).

Ibuprofen, salbutamol, and atenolol stock solutions were prepared in MeOH to obtain a final concentration of 200 $mg \cdot L^{-1}$. Analyte stock solutions were stored in the dark at 4 °C for 3 months maximum. Working standard solutions were prepared daily by appropriate dilution of stock solutions in ACN as required.

Different mixtures of ACN–ammonium acetate buffer 5 mM pH 6 (95:5 (v/v), 75:25 (v/v)) were used as mobile phase.

2.2. Chromatographic Equipment

A Jasco LC-NetII/ADC LC chromatograph (JASCO Analitica Spain, Madrid, Spain), consisting of a degasser, a Jasco PU-2089 Plus quaternary gradient pump (JASCO Analitica Spain, Madrid, Spain), and a Jasco Md-2018 Photodiode Array Detector (JASCO Analitica Spain, Madrid, Spain), was employed for chromatographic analyses. An external stainless-steel loop with a volume of 20 µL was placed into a manual Rheodyne® injection valve (LC CONNECTION COMPANY, Cotati, CA, USA). Columns evaluated in this work were Kinetex HILIC (2.6 µm, 2.1 × 150 mm, 100 Å), Atlantis C18 (5 µm, 4.6 × 150 mm, 100 Å), and LUNA C18 (5 µm, 4.6 × 150 mm, 100 Å), all supplied by Phenomenex (Torrance, CA, USA).

Nylon filters of 0.45 µm pore size (Sigma-Aldrich, Barcelona, Spain) were used for filtration of mobile phases.

2.3. Equipment and Materials for Sample Preparation

For preparation of urine samples and solutions, a pH-meter and vortex, both from VELP Scientifica (Usmate Velate, MB, Italy), an ultrasonic bath and membranous vacuum pump from J. P. Selecta (Barcelona, Spain), and a MiniVap 6 Port vacuum concentrator from Supelco (St. Louis, MO, USA) were employed.

Sample SPE in cartridges was carried out by a VacElut 20-place vacuum manifold supplied by Varian (Harbor City, CA, USA) connected to a Selecta membrane vacuum pump (Barcelona, Spain). Sep-IC-H® (polymeric strong cation exchanger, 450/300 mg, 0.5 mL) cartridges from Lida Manufacturing Corporation (New York, NY, USA), DSC-C18 (500 mg, 6 mL), and HLB ExtraBond® Polymeric (polystyrene divinylbenzene modified

with pyrrolidone, 200 mg, 6 mL) cartridges, both from Scharlab (Barcelona, Spain), were used for preconcentration and sample clean-up.

2.4. Experimental Procedures

2.4.1. HILIC Chromatographic Determination

Chromatographic separation was performed on a Kinetex HILIC (2.6 µm, 2.1 × 150 mm, 100 Å). The mobile phase consisted of ACN (A) and aqueous ammonium acetate buffer 5 mM pH 6 (B). The initial composition was 95%A and 5%B. The composition was maintained for 2 min, and then a gradient elution was carried out where the phase B increases linearly to 25% during 7 min and the mobile phase composition was changed to the initial one to re-equilibrate the column for the next injection.

Separation was performed at room temperature; the flow rate was set to 0.40 $mL \cdot min^{-1}$ and the injection volume was 20 µL The chromatographic system was connected to a Jasco DAD detector and the quantification was performed at 227 nm for ibuprofen and at 275 nm for both atenolol and salbutamol.

2.4.2. Analytical Characteristics of the HILIC-LC Method

The method performance of the chromatographic method developed was evaluated in terms of linear range, precision, and sensitivity. External calibration curves (n = 5) were obtained from 0.05 to 4.40 $\mu g \cdot mL^{-1}$ for ibuprofen, and in the range 0.02 to 5.00 $\mu g \cdot mL^{-1}$ for both salbutamol and atenolol.

Detection (LOD) and quantification limits (LOQ) were estimated at a signal/noise ratio of 10/3 and 10, respectively. Precision of both retention factors and peak areas were evaluated by injection of standard solutions, at a concentration of 2 $\mu g \cdot mL^{-1}$, in the same day (intra-day variation, n = 3) and at different days (inter-day variation, N = 9).

2.4.3. SPE Protocols

SPE procedures for extraction of target analytes from evaluated cartridges were exhaustively evaluated and optimized. Different sorbents and SPE procedures were tested to optimize the extraction procedure and achieved acceptable recoveries for all the analytes simultaneously. In these studies, spiked water solutions and/or spiked diluted urine were employed. Blank water and urine samples were processed in the same way that spiked solutions were processed to control coextraction of endogenous matter and possible interferences.

For the study with the DSC C18 cartridges, the procedure described by Magiera et al. [13] was followed with slight modifications. DSC-C18 cartridges were conditioned with 5 mL of methanol and 5 mL of diluted formic acid (0.01 % (v/v) pH 4). A volume of 25 mL of diluted urine in a 1:50 (v/v) ratio, spiked at a concentration level of 0.06 $\mu g \cdot mL^{-1}$ for each analyte, was passed thorough the sorbent. After retention, a washing step was accomplished with 5 mL of diluted formic acid (0.01 % (v/v), pH 4). Then, 5 mL of 5% (v/v) ammonium hydroxide in methanol was employed as elution solution. The eluted extracts were evaporated to dryness under a nitrogen stream and reconstituted with 0.5 mL of acetonitrile. HPLC analysis was performed according to the procedure described in Section 2.4.1.

Regarding Sep-IC-H® cartridges, 5 mL of water acidified with H_3PO_4 4% (v) at pH 2 and spiked at 2 $mg \cdot L^{-1}$ $\mu g \cdot mL^{-1}$ of each analyte was used for retention studies. Cartridges were previously conditioned with 0.50 or 2.0 mL of both methanol and water. H_3PO_4 4% (v) and HCl 0.1 M both at pH 2 were also employed as conditioning solutions instead of water. After sample loading, cartridges were rinsed with 0.50 or 1.0 mL of water, formic acid 2% (v), HCl 0.1 M, or HCl 0.05 M. Then, elution was accomplished with 0.5–3.0 mL of both methanol and 5% (v/v) ammonium hydroxide in methanol. Obtained extracts

were evaporated to dryness under a gentle nitrogen stream at 60 °C. Finally, they were reconstituted with 5 mL of acetonitrile before LC-UV analysis. A total of 20 µL of the resulting solution was injected into the chromatographic system and analyzed under optimum conditions described above for the HILIC column.

Analogous to Sep-IC-H® cartridges, several assays were performed to optimize the extraction conditions on the HLB ExtraBond® ones. After evaluation of the recovery results obtained from both studied cartridges, ExtraBond® cartridges were selected as the optimum for the SPE of ibuprofen, salbutamol, and atenolol from urine samples. The best results were obtained by using 1.0 mL of MeOH and 1.0 mL of aqueous solution of HCl 0.1 M pH 2 as conditioning solutions, 1.0 mL of a mixture of methanol–water 5:95 (v/v) as washing solution to eliminate inferences, and 2.0 mL of MeOH and 1.0 mL of 5% (v/v) ammonium hydroxide in methanol as elution solvents. The eluted volume (3 mL) was evaporated to 0.15 mL under nitrogen stream at 60 °C, and it was reconstituted to 5 mL with acetonitrile. A volume of 20 µL of the resulting solution was analyzed by the developed HILIC LC-UV method.

In all tested procedures, extraction recoveries were established by comparing concentrations in triplicate calculated from calibration curves with the spiked amounts.

2.4.4. Analysis of Spiked Urine Samples

Urine samples were prepared by diluting the commercial urine in a 1:10 (v/v) ratio with Milli Q aqueous solution of HCl 0.1 M at pH 2. Then, 20 µL of each analyte stock solution were added to obtain a final concentration of 0.4 $\mu g \cdot mL^{-1}$. Spiked urine samples (10 mL) were passed through the HLB ExtraBond® cartridges previously conditioned. Washing and elution steps were performed as described in Section 2.4.3. After evaporation, reconstitution was made to a final volume of 2 mL with acetonitrile. Finally, 20 µL were injected into the chromatograph for identification and quantification purposes.

Extraction recoveries were established comparing concentrations calculated from calibration curves with the initial added amount. Accuracy was expressed as % RSD of the recoveries at the studied spiked level.

3. Results and Discussion

3.1. Optimization of Chromatographic Conditions

3.1.1. Preliminary Assays

As described in the literature, the separation of salbutamol and atenolol is possible using acetonitrile–water elution gradients with ammonium acetate at pH 6.8 [3], and the separation of salbutamol, atenolol, and ibuprofen with acetonitrile–water gradients with 0.1% formic acid (salbutamol and atenolol) or with 4 mM ammonium acetate (ibuprofen) [4], or with gradients with methanol–water mixtures containing formic acid/formate at pH 3.2 (salbutamol and atenolol) or with gradients with methanol ammonium acetate (ibuprofen) [5] using C18 columns. Considering the acid–base characteristics of the compounds (Figure 1), the first separation assays of these three analytes were performed with mobile phases containing a buffer at pH 4 in a C18 reversed-phase Atlantis column. This stationary phase contains di-functionally bonded C18 ligands, that have been optimized for use with highly aqueous mobile phases. The mobile phases used were MeOH mixtures–aqueous solution of 5 mM acetate acetic acid buffer at pH 4 and ACN mixtures–aqueous solution of 5 mM acetic acid acetate buffer at pH 4 in proportions between 80 and 90% of organic modifier. The separation of the three compounds was only possible with a mobile phase 90:10 (v/v) ACN–aqueous solution of 5 mM acetate acetic acid buffer at pH 4 with retention times of 4.9 min for ibuprofen, 14.3 min for atenolol, and 17.9 min for salbutamol. The last two chromatographic peaks had considerable width, and the retention

times and shape of the peaks could not be improved by applying changes in the mobile phase composition. Subsequent assays are performed with a Luna C18 column that is designed to improve the shape of the chromatographic peaks, but it did not allow us to obtain an adequate separation of the three analytes under study when ACN ratios between 20 and 90% were used.

Taking into account the polar character of the analytes evaluated, ibuprofen contains a carboxylic acid group with a pKa of 4.4 [22]; salbutamol has two ionizable groups, one due to the phenol group (pKa 9.4) and the second due to a secondary amine (pKa 10.3) [23]; and atenolol also presents a secondary amino group with a pKa of 9.6 [24]. Consequently, the most appropriate way to separate them would be to use a HILIC ion interaction mechanism.

HILIC columns have already been used successfully for the separation of salbutamol and atenolol using a Kinetex HILIC column in isocratic mode and a ternary mobile phase MeOH–ACN–ammonium acetate buffer 5 mM at pH 6 [10], to separate ibuprofen from other nonsteroidal anti-inflammatory drugs in plasma [14] with a UK-Amino HILIC column using an elution gradient with acetonitrile and an aqueous solution of ammonium acetate at pH 6.8, or to separate ibuprofen from other acidic compounds [15] using a ZIC-HILIC column with an elution gradient with acetonitrile and 10 mM ammonium acetate. The stationary phases used (bare silica and amino propyl) favor electrostatic interactions between the analytes and the stationary phase [14], while the amino or ZIC phases seem more suitable for compounds with an acidic character [14,15]. The silica-based phases can be suitable for both acids and bases, so, for the separation of the analytes of interest, a Kinetex HILIC column was chosen.

To promote both the partition-controlled retention process and the electrostatic interactions, it is necessary that we evaluate both the proportion of acetonitrile and the pH of the aqueous solution in the mobile phase. Using mobile phases composed of 70:30 (*v*/*v*) acetonitrile–2 mM ammonium acetate buffer at pH 4, the separation of salbutamol and atenolol is obtained with retention times of 10.0 min and 14.5 min, respectively, while ibuprofen under these conditions is eluted with dead time.

In order to obtain the adequate retention of ibuprofen, mobile phases with a high content of acetonitrile, 95% (v), and solutions of 5 and 10 mM ammonium acetate at pH 6 were used. Higher buffer concentrations produce a splitting of the ibuprofen chromatographic peak, probably because both the protonated and deprotonated forms are in equilibrium in solution, with a buffer concentration of 5 mM at pH 6, a single peak with a retention time of 2.5 min is obtained. Different proportions of acetonitrile were evaluated to separate the three compounds in an isocratic way, even the possibility of using ternary mobile phases such as those used in the work of Yung et al. [10]; 5:90:5 (v/v/v) ACN–MeOH buffer was evaluated, but in no case were satisfactory separations obtained in short periods of time. Therefore, various elution gradients were evaluated, so that, using an initial composition 95:5 (*v*/*v*) ACN–ammonium acetate buffer 5 mM at pH 6 during the first two min, and using a linear gradient for 7 min up to a composition of 75:25 (*v*/*v*) ACN–ammonium acetate buffer 5 mM at pH 6, the separation of the three compounds is obtained in a time of less than 10 min and with baseline separation for all compounds.

3.1.2. Analytical Characteristics

The developed method has been validated in terms of the linearity range, detection and quantification limit, and reproducibility following the validation methodology described by Rambla-Alegre et al. [25]. The accuracy of the method has been estimated using standards of the same concentration (2 $\mu g \cdot mL^{-1}$) on the same day and on three different days. The repeatability and instrumental precision were evaluated by calculating the relative standard deviation of retention factors (k) and peak areas; the intra-day precision was evaluated from

three consecutive injections, and inter-day variation from three different days (Table 1). The detection and quantification limits were calculated as 3.3 and 10 times the background noise signal. All measures were taken at a wavelength of 227 nm for ibuprofen and 275 nm for atenolol and salbutamol. The wavelengths selected for quantification correspond, in the case of ibuprofen, to its maximum absorption at 227 nm (Figure S1), while, for atenolol and salbutamol, the wavelength corresponding to a second maximum of absorption, fixed at 275 nm (Figure S1), was selected since the elution gradient used presents a very pronounced baseline at 227 nm (Figure 2a); Table 1 includes the main analytical characteristics of the developed method, with linear ranges suitable for the three analytes (R^2 between 0.994 and 0.996) and with inter-day coefficients of variation between 4.5 and 10.9% for the peak areas and in the range of 1.3–5.4% for retention factors. The highest irreproducibility was observed for ibuprofen, which is the least retained compound and closest to the dead time. A higher sensitivity is observed for salbutamol and atenolol, which are the compounds with the lowest detection and quantification limits.

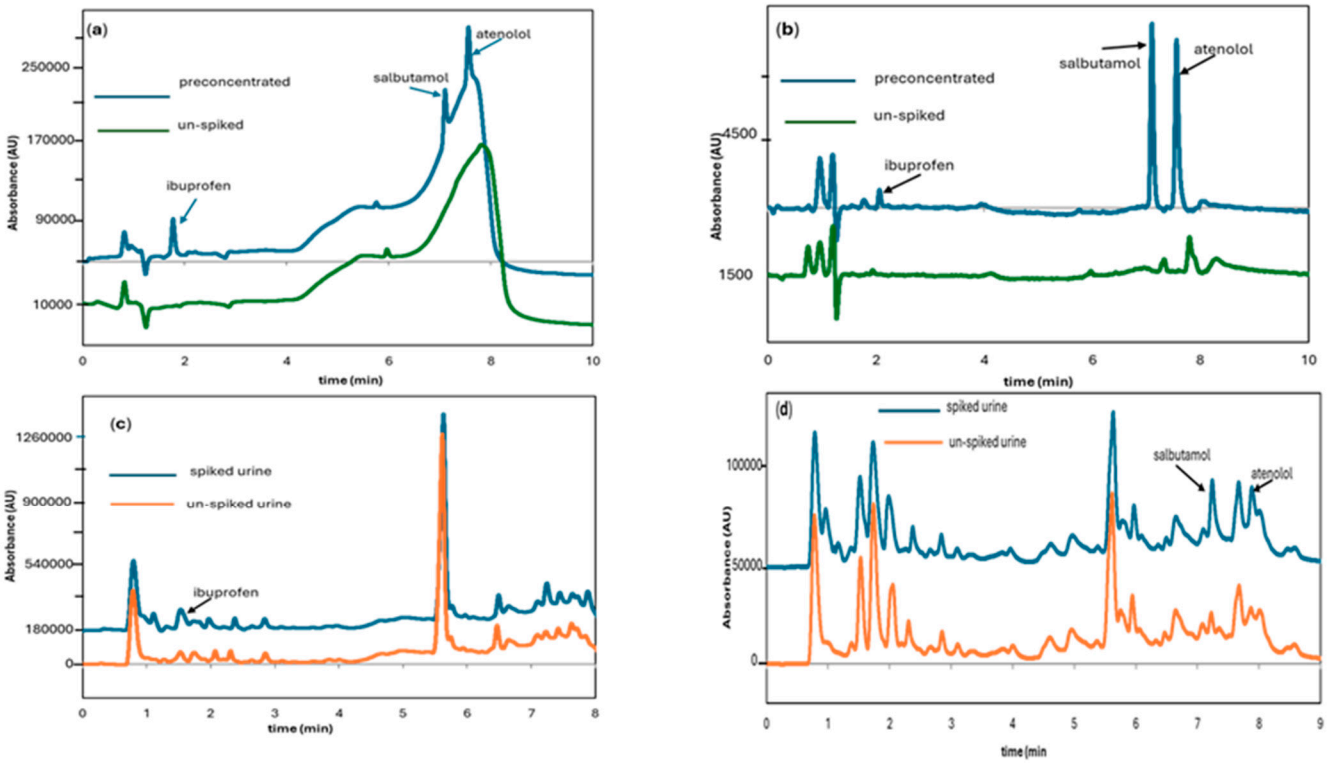

Figure 2. HILIC-DAD chromatograms registered at 227 nm (**a**,**c**) and 275 nm (**b**,**d**) for the separation of preconcentrated mixtures of ibuprofen, salbutamol, and atenolol on the Extrabond® cartridge: (**a**,**b**) aqueous solution spiked at 0.40 mg·L^{-1} concentration level for each analyte and preconcentrated un-spiked aqueous solution; and (**c**,**d**) spiked preconcentrated urine with 0.40 mg·L^{-1} of salbutamol, ibuprofen and atenolol and un-spiked preconcentrated urine. The scale shown on the y axis of (**a**,**b**) orresponds to the signal of un-spiked sample, while the scale of the chromatogram of the preconcentrated sample is the scale of un-spiked plus 40,000 and 3000 absorbance units (AU), respectively. The scale shown on the y axis of (**c**,**d**) corresponds to the signal of un-spiked urine sample, while the scale of the chromatogram of the spiked urine sample is the scale of un-spiked urine plus 180,000 and 50,000 absorbance units (AU), respectively. AU: arbitrary units.

Table 1. Analytical characteristics of the HILIC-DAD method.

Compound	LOD/LOQ ($\mu g \cdot mL^{-1}$)	Linear Range ($mg \cdot L^{-1}$)	Calibration Curve			CV (%) Intra-Day (n = 3)		CV (%) Inter-Day (N = 9)	
			a *	b ** ($mL \cdot \mu g^{-1}$)	R^2	k	Area	k	Area
ibuprofen	0.025/0.084	0.05–4.40	$(2 \pm 1) \cdot 10^5$	$(171 \pm 4) \cdot 10^3$	0.996	1.7	9.3	5.4	10.9
salbutamol	0.007/0.022	0.02–5.00	$(35 \pm 4) \cdot 10^3$	$(14 \pm 7) \cdot 10^3$	0.994	1.1	3.3	1.8	4.5
atenolol	0.008/0.028	0.02–5.00	$(25 \pm 3) \cdot 10^3$	$(9 \pm 6) \cdot 10^3$	0.996	0.4	5.0	1.3	6.1

where a * intercept, and b ** slope of the regression equations.

3.2. SPE Preliminary Assays

Firstly, DSC-C18 cartridges, based on reversed-phase retention mechanisms, were evaluated, as described in Section 2.4.3, with spiked urine samples. A HCl 0.1 M solution was also tested as a washing solution, although the recoveries obtained did not improve compared to diluted formic acid (0.01% (v/v)) at pH 4. Additionally, the convenience of removing the first eluted 0.5 mL was observed to decrease the amount of co-eluted endogenous matter. However, under optimum conditions, only ibuprofen and salbutamol could be determined with recoveries of (75.9 ± 0.9)% and (28 ± 2)%, respectively. Regarding atenolol, the high recoveries estimated (246 ± 9)% suggested a possible co-elution with endogenous matter. The results obtained differ from those obtained by other authors who used C18-modified mesoporous silica [4] and presented recoveries in the range of 53–67% for salbutamol, 76–85% for atenolol, and 56–57% for ibuprofen in meat samples. Although the results for ibuprofen are acceptable, the values obtained for salbutamol and atenolol are not adequate for the objective of this work, and, then, other SPE cartridges exhibiting different retention mechanisms were evaluated.

Various authors have used polymeric cartridges using mixed-mode SPE to retain very polar substances using different conditions of retention, washing, and elution [3,10]. Several extraction conditions were evaluated with polymeric Sep-IC-H® cartridges, which act as a strong cation exchanger (Section 2.4.3). In general, low recoveries were obtained for ibuprofen in all tested conditions. The maximum recovery obtained, around 44%, was estimated when the cartridge was conditioned with 0.5 mL of both methanol and water, the washing step was performed with 0.5 mL of 2% (v) formic acid solution, and the elution was consecutively made with 0.25 mL of methanol and 0.25 mL of 5% (v/v) ammonium hydroxide in methanol. Regarding atenolol and salbutamol, the maximum recoveries, around 54 and 100%, were, respectively, obtained when the cartridge was conditioned with 2 mL of both methanol and 4% (v) H_3PO_4 acidified water, washed with 0.5 mL of 2% (v) formic acid solution and eluted with 3 mL of methanol and 3 mL of 5% (v/v) ammonium hydroxide in methanol. To increase the recoveries and to find compromise conditions for the simultaneous extraction of the three target analytes, some attempts were carried out by changing the nature and the volumes of the conditioning, washing, and eluted solutions. The best recoveries were obtained by employing 2 mL of methanol and 2 mL of HCl 0.1 M at pH 2 as conditioning solutions, 0.5 mL of water for the washing step, and 1.0 mL of methanol and 3 mL of 5% (v/v) ammonium hydroxide in methanol as elution solutions. Under these extraction conditions, the calculated recoveries for both salbutamol and atenolol were higher than 90%, similar to those found for other authors [10], although very low recoveries, around 15%, were achieved for ibuprofen. It was also observed that ibuprofen was totally eluted in the first collected fraction of methanol, while atenolol and salbutamol were eluted in the methanolic ammonium hydroxide fraction. In addition, the evaporation to dryness of the eluted extracts negatively affected the stability and volatility of the analytes. Therefore, they were evaporated to a final volume of 0.15 mL under a nitrogen stream at 60 °C.

To retain both acidic and basic compounds, a hydrophilic–lipophilic balanced (HLB) mechanism has finally been used, which provides retention for many compounds [5]. Polymeric HLB ExtraBond® cartridges were employed to increase the recovery obtained for ibuprofen. The conditioning step was tested by using 1 mL of methanol, 1 mL of water, and 1 mL of 1:1 (*v*/*v*) HCl 0.1 M solution. The washing stage was performed with 1 mL of 5% (v) methanolic aqueous solution. For elution, methanol volumes between 1 and 2 mL, and 1 mL or 1.5 mL of 5% (*v*/*v*) ammonium hydroxide in methanol were evaluated. The maximum recoveries were obtained when 2 mL of methanol and 1.5 mL of 5% (*v*/*v*) ammonium hydroxide methanolic solution were consecutively used as elution solvents. Under optimum conditions, recoveries around 100% were obtained for atenolol, while recoveries of (52 ± 2)% and (55 ± 4)% were estimated for ibuprofen and atenolol, respectively (Figure 2a,b).

With the aim of establishing the maximum aqueous volume that can be percolated through the HLB ExtraBond® sorbent, volumes between 5 and 25 mL of acidified HCl 0.1 M water at pH 2 containing 0.40 mg·L^{-1} of each analyte were passed through the cartridges. A considerable decrease in the recovery was observed for salbutamol when 25 mL of the aqueous spiked solution were percolated. Consequently, a maximum volume of the sample of 10 mL should be passed thorough the cartridge to avoid losses of the three studied analytes.

3.3. Analysis and Recovery Study from Spiked Urine Samples

After SPE previous studies with spiked water solutions, it was concluded that only HLB ExtraBond® cartridges provided acceptable recoveries for the basic analytes, also increasing the recovery of the ibuprofen acid drug. In fact, when DSC-C18 cartridges were employed with spiked urine samples, recoveries around (76 ± 1)% were determined for ibuprofen, but very low recoveries (28 ± 2)% were estimated for salbutamol, and atenolol was co-eluted with endogenous organic matter. Regarding MCX, although recoveries of (63 ± 10)% and (72 ± 9)% were obtained for salbutamol and atenolol, respectively, low recoveries were observed for ibuprofen (29 ± 2)%.

Consequently, to evaluate the performance of the selected cartridge for the simultaneous drug determination in urine samples, commercial urine was diluted with water in a 1:10 ratio. Then, it was spiked by triplicate with the appropriate volume of each analyte stock solution to reach a concentration level of 0.40 mg·L^{-1}.

The SPE procedure was applied according to the optimum conditions described in Section 2.4.3. A urine blank was also prepared and analyzed in the same conditions as samples, not observing interfering peaks of endogenous matter at the retention times of the analytes studied (Figure 2c,d).

Extraction recoveries were estimated by a comparison of the concentrations calculated from calibration curves with the initial added amounts to dilute urine (Table 2). The recoveries obtained were (74 ± 8)% for ibuprofen, similar to those found for other authors [13], (63 ± 9)% for salbutamol, and (96 ± 8)% for atenolol. The three analytes have been preconcentrated with the same SPE cartridge, while, in the literature consulted, the preconcentration protocols are common for salbutamol and atenolol but different for ibuprofen. The three compounds have been preconcentrated simultaneously in meat and wastewater samples (Table 2) and the recoveries obtained are comparable to those obtained with the protocol optimized for the HLB Extrabound cartridge used.

As can be observed, the recoveries obtained for the spiked urine samples were acceptable for all analytes. They were comparable and even better than those obtained for the spiked water solutions under the same extraction conditions, showing the efficiency of the selected cartridges for the simultaneous determination of basic and acid drugs in

this biological complex sample. In addition, the recovery SD values were lower than 10%, showing the high precision and repeatability of the developed protocol which allows us to determine the low amounts of these drugs that can be excreted in the urine considering the doses at which they are administered [1,8,11].

Table 2. Comparison between the proposed SPE method and other methods for ibuprofen, atenolol, and salbutamol.

SPE Cartridge	Sample	Analyte	Recovery (%)	Repeatability (%)	Reference
Isolute HCX	urine	Salbutamol Atenolol	84–88 85–90	2–4 4–8	[10]
Isolute IST	urine	salbutamol atenolol	33 55	- -	[7]
Oasis MCX	urine	ibuprofen	80.5	12.9	[13]
Oasis MCX	surface water	atenolol salbutamol	90 88	-	[7]
Oasis MCX	urban waste	atenolol ibuprofen salbutamol	106 92 76	6 3.7 4.4	[9]
Oasis HLB	urine	ibuprofen	75.2	15.09	[13]
Oasis HLB	Surface water	atenolol	98–123	-	[7]
Octadecyl C18	urine	ibuprofen	79.18–88.4	0.01–9.58	[13]
C18-modified mesoporous silica	meat	salbutamol atenolol ibuprofen	53–67 75–85 56–57	4-4–19.5 1.6–11.7 8.6–16.2	[4]
HLB Extrabound	urine	ibuprofen salbutamol atenolol	74 63 96	8 9 8	Proposed method

4. Conclusions

This study has focused on the simultaneous extraction and determination of free ibuprofen, salbutamol, and atenolol in urine samples at low concentration levels, which are expected when these drugs are consumed (21–24). Separation using hydrophilic interaction liquid chromatography is an alternative to HPLC separation with reversed-phase columns. Separation is possible in a single chromatogram in less than 10 min. The pH control of the mobile phase is essential for promoting secondary interactions that facilitate separation. The acid–base nature of the substances studied has made it necessary to exhaustively evaluate the sample preparation conditions using solid-phase extraction with stationary phases, hydrophilic–lipophilic balanced, with the retention of the analytes at an acidic pH and elution with methanol at a basic pH, which has allowed the recovery of the analytes in a single fraction with acceptable recoveries. The proposed methodology simplifies the determination of ibuprofen, salbutamol, and atenolol in urine samples, both by the selected chromatographic separation and by the sample treatment with the solid-phase extraction used.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/separations12020026/s1, Figure S1: UV–Vis spectra registered for 5.0 mg·L^{-1} acetonitrile solutions of ibuprofen, salbutamol, and atenolol.

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