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A novel application of immobilization on membranes for the separation and spectrofluorimetric quantification of amiloride and furosemide in pharmaceutical samples

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ABSTRACT

A new, simple and highly sensitive method for spectrofluorimetric determination of amiloride (AMI) and furosemide (FUR) in pharmaceuticals is presented. The proposed method is based on the separation of AMI from FUR by solid-phase extraction using a nylon membrane, followed by spectrofluorimetric determination of both drugs, on the solid surface and the filtered aqueous solution, respectively. AMI shows low native fluorescence, but its separation-preconcentration by immobilization (solid-phase extraction) on nylon membrane surface provides a considerable enhancement in fluorescence intensity. The fluorescence determination is carried out at λ_{ex} = 237, λ_{em} = 415 nm for FUR; and λ_{ex} = 365, λ_{em} = 406 nm for AMI. The calibration graphs are linear in the range 3.20×10^{-4} to 0.8 μg mL^{-1} and 1.33×10^{-3} to 4.0 μg mL^{-1}, for AMI and FUR, respectively, with a detection limit of 9.62×10^{-5} and 4.01×10^{-4} μg mL^{-1} ($S/N = 3$). The commonly found excipients in commercial pharmaceutical formulations do not interfere. The developed method is successfully applied to the determination of both drugs in pharmaceutical formulations.

1. Introduction

Amiloride hydrochloride and furosemide are widely used in different types of diuretics. These pharmaceutical formulations are supplied in numerous therapeutical indications, such as arterial hypertension, cardiac insufficiency, and hepatic cirrhosis since they rise the rate of urine formation, increasing the excretion of electrolytes, especially sodium, chloride and water. The main organ on which diuretics act is the kidney. There, their components interfere in the re-absorption of sodium and other ions [1,2].

AMI (N-amidino-3,5-diamino-6-chloropyrazine-2-carboxamide) (Fig. 1) has the property of interfering with the process of cationic interchange in the distal tube [1]. It blocks the absorption of sodium ions and the excretion of potassium ions.

FUR (4-chloro-N-furfuryl-5-sulfamoylanthranilic acid) (Fig. 1) acts inhibiting the co-transportation of sodium, potassium and chloride, and further causes excretion of calcium, magnesium and bicarbonate ions [1].

Individual determinations of AMI in pharmaceutical formulations and in biological fluids have been reported, utilizing isopotential fluorimetry [3], capillary zone electrophoresis with fluorescence detection [4] and electrochemical techniques [5].

On the other hand, methods for FUR determination described in the literature involve spectrophotometric analysis in pharmaceutical formulations [6,7], and HPLC [8] and HPLC–mass spectrometric analysis, in biological fluids [9].

Some analytical methods for the determination of AMI or FUR in presence of other drugs in pharmaceutical formulations and/or in biological fluids [10,11] have also been reported. For instance, the determination of FUR in tablets and urine by HPLC with amperometric detection [12,13], by micellar electrokinetic chromatography [14] and by reversed phase-high performance liquid chromatography [15,16].

Although, AMI and FUR are usually found in commercial pharmaceutical formulations containing both drugs, in the literature there are only a few reports about its simultaneous determination. The determination of both drugs in urine samples, using isocratic reversed phase chromatography (RPLC) [17] and by HPLC using a micellar mobile phase of sodium dodecylsulfate [18], have been described. Toral and Pope described a digital derivative spectrophotometry method for determination of both drugs in pharmaceuticals [19]. This last method did not propose a separation of the drugs, but obtains low detection limits. The US pharma-

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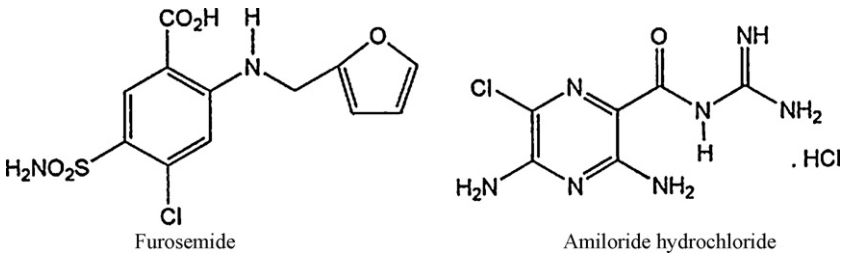

Fig. 1. Chemical structures of furosemide and amiloride hydrochloride.

copoeia [20] does not present a method to determine them in compound formulas. The necessity of a precise quality control of both drugs in finals products or in bulk requires the development of sensitive and selective analytical methodologies.

In this work, a simple method to separate and precisely determine both drugs, is proposed. AMI and FUR present an important fluorescence spectral interference. The approach is to separate AMI, extracting it from the sample matrix by chemofiltration with a nylon membrane, while FUR is maintained in the filtered aqueous solution. The optimization of filtration variables such as pH, flow rate, solvent nature, as well as the fluorescence parameters, were studied. The proposed method was successfully applied to commercial pharmaceutical formulations that contain both drugs.

2. Experimental

2.1. Apparatus

A Shimadzu RF-5301PC spectrofluorimeter (Shimadzu Corporation, Analytical Instrument Division, Kyoto Japan), equipped with a Xenon discharge lamp, 1 cm quartz cells and solid sample holder with quartz plate were used for the fluorescence measurements.

Polyamide membrane filters Millipore HNWP04700 (USA) and Gamafil Y04-047-BLNC (Argentine Industry) of 0.45 μm pore size were used for filtering. An in line Millipore polypropylene filter holder of 47 mm i.d., with closing nylon butterflies and silicon toric joint was used. Solutions were propelled through the filter holder using a Gilson Minipuls 3 peristaltic pump fitted with rate selectors and PVC tubing of 0.8 mm i.d.

A pHmeter (Orion Expandable Ion Analyzer, Orion Research, Cambridge, MA, USA) Model EA 940 with combined glass electrode was used for monitoring pH adjustment.

2.2. Reagents

AMI and FUR were kindly provided by Lab. Puntanos, San Luis, Argentina.

The pH values were adjusted by adding NaOH 0.01 mol L^{-1}, NaOH (c) (Mallinckrodt Chemical Works, New York, Los Angeles, St. Louis, USA), HCl 0.01 mol L^{-1} or HCl (c) (Merck, Darmstadt, Germany) until the desired pH was reached.

Standard solutions: 1 mg mL^{-1} of AMI (MW 302.1) and 1 mg mL^{-1} of FUR (MW 330.7) were prepared by dissolving 25 mg of each compound in 25 mL of ultra pure water and 25 mL of NaOH 0.1 mol L^{-1}, respectively. In these conditions, both solutions are stable for 7 days, when are kept in dark. Working solutions were prepared daily by appropriate dilution of the standard solutions using the same solvents.

Sample solutions: Five tablets of Nuribán-A® (Lab. Roux-Ocefa, Bs. As., Argentina), Errolón-A® (Lab. Sandoz, Bs. As., Argentina) or Lasiride® (Lab. Aventis, Bs. As., Argentina), labeled to contain 5 mg AMI and 40 mg FUR per tablet, were weighted and finely powdered. A portion of the powder, equivalent to 5 mg of AMI, was accurately weighted and dissolved in 80 mL of NaOH 0.1 mol L^{-1} and filtered to remove insoluble material. The filtered solution was transferred to a 100 mL volumetric flask and taken to volume with ultra pure water. The final concentration was about 50 μg mL^{-1} of AMI and 0.4 mg mL^{-1} of FUR. Further dilutions were done with ultra pure water.

All other chemicals used were of analytical grade and ultra pure water was used throughout.

2.3. General procedure

2.3.1. Separation-preconcentration process and fluorescent determination of AMI on solid substrate

Polyamide membranes, without previous conditioning, were placed in Millipore filter holder and 10 mL of solutions containing AMI or sample at pH 11 were filtered through it using a positive pressure, keeping a flow rate of approximately 4 mL min^{-1}. Filtered solutions were reserved. After the membrane was dried, the disc was placed in a solid sample holder, and the fluorescence spectrum was scanned. The excitation and emission wavelengths were adjusted at 365 and 406 nm, respectively. In all cases, the excitation and emission slit widths were of 1.5 nm.

2.3.2. Fluorescent determination of FUR in aqueous solution

For FUR determination, the filtered solutions were obtained as was described in Section 2.3.1 procedure, and the pHs of solutions were adjusted at 2.7 with HCl. Then, sample and standards solutions were introduced into the spectrofluorometer and the fluorescent emission was measured at λ_{em} = 415 nm using λ_{ex} = 237 nm. In all cases, the excitation and emission slit widths were 3 and 5 nm, respectively.

2.4. Validation procedure

In order to demonstrate the validity of this methodology, an accurate weight of commercial tablets powder containing 5 mg AMI and 40 mg FUR, were dissolved and transferred to a 100 mL volumetric flask and taken to volume with water. 20 μL portions of this sample solution were transferred to 10 volumetric flasks of 10 mL. The developed methodology was applied to six portions and the average quantity of AMI determined was taken as a base value. Then, increasing quantities of AMI were added to other four aliquots of sample and AMI concentrations were determined, applying the addition standard method. All samples were taken to volume with water.

The same validation procedure was applied for FUR, but in this case, using the filtered sample solutions.

3. Results and discussion

3.1. Fluorescent characteristics of AMI

The excitation and emission fluorescent spectra obtained for the analyte supported on a solid substrate and in aqueous solution can be seen in Fig. 2. As shown, the obtained spectral intensities

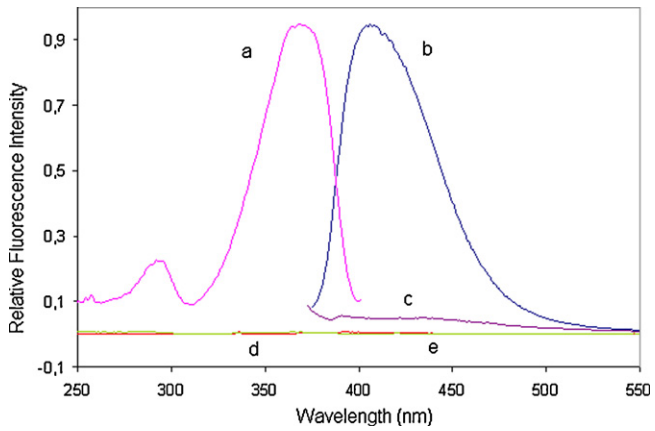

Fig. 2. Excitation (a) and emission (b) fluorescence spectra for AMI (0.8 μg mL^{-1}) on a nylon membrane at pH 11 (λ_{ex} = 365 nm). Emission (c) fluorescence spectra of nylon membrane (blank). Excitation (d) and emission (e) fluorescence spectra for AMI (0.8 μg mL^{-1}) in solution. Slit widths 1.5 nm.

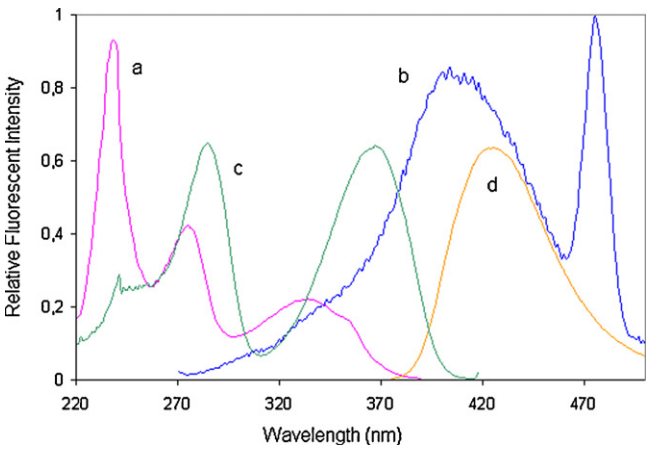

Fig. 4. Excitation (a) and emission (b) spectrum of FUR (0.16 μg mL^{-1}) at pH 2.7 (λ_{ex} = 237 nm and λ_{em} = 415). Excitation (c) and emission (d) spectrum of AMI (0.16 μg mL^{-1}) at pH 11 (λ_{ex} = 365 nm and λ_{em} = 424). Slit width ex: 5 nm, em: 10 nm.

for AMI aqueous solution 0.8 μg mL^{-1} and the same quantity of AMI on a polyamide membrane, both at pH 11, are quite different. When AMI is immobilized on the polymide membrane, the rigidity enhancement in the molecule produces a highly favourable condition for the fluorescence emission, showing high intensity spectra. Whereas, with AMI aqueous solutions, having the same concentration and are measured with the same slit widths (1.5 nm), fluorescence practically cannot be observed. Additionally, the preconcentration produced by the chemofiltration process gives a beneficial enhancement of the fluorescence signal. It is also important to observe in Fig. 2 the absence of fluorescence signals in the blank.

The drug AMI immobilized on the membrane shows a maximum emission wavelength at 406 nm when it is excited at 365 nm. The spectrum of AMI on the solid support compared to that in solution, Fig. 2, indicates that the polyamide support does not promote significant changes in the fluorescent behavior of AMI. The shape of the spectrum is preserved, but, a slight shift in maximum wavelengths from 424 to 406 nm, is observed. It is notable, the increase in the fluorescence signal of AMI on the solid support, even though the concentration of AMI in solution is rather higher.

3.2. Effect of pH on AMI immobilization and fluorescence intensity

In order to determine the optimal pH for the AMI separation, series of standard working solutions at different pH values were evaluated. Each operational desired pH value was obtained conditioning the solutions with diluted HCl and/or diluted NaOH. These systems were processed as was described in general analytical procedure.

The knowledge of the possible ionization states of the analyte in the working conditions is essential either for the adsorption or elution from the solid support. On the non-polar polyamide membrane, AMI molecule must stay in its neutral form for achieving quantitative retention. In agree with the acidity constant value of AMI (pK_a = 8.7) [21], in basic solution, hydroxyl ions may react with AMI to produce neutral species, which can be retained on the membrane [5]. Fig. 3 shows the effect of pH of medium, respect to the immobilization, through the fluorescent signal of AMI on both matrixes, aqueous and polyamide. The working pH selected for filtration was 11 due to the highest intensity obtained on solid substrate, indicating a maximum quantity of AMI retained.

3.3. Effect of flow rate on AMI retention

In this experiment the AMI solutions were on-line filtered through the polyamide membrane using different flow rates.

Several experiments were made in order to study the effect of flow rate of filtration on fluorescent signal. The results showed that the fluorescent signal increases with the increase of flow rate. Then, a flow rate of 4 mL min^{-1} was selected as optimal. Thus, the

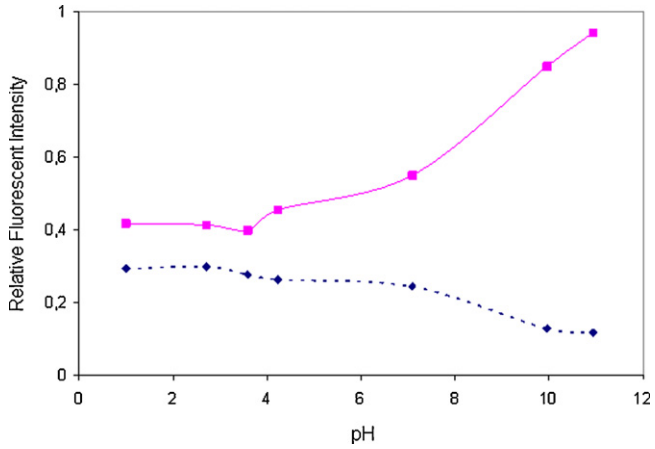

Fig. 3. Effect of pH on AMI fluorescent intensity: AMI initial concentration 0.02 μg mL^{-1}. (- - -) AMI solution after filtering. Slit widths 10 nm. (—) AMI retained on polyamide membranes. Slit widths 3 nm.

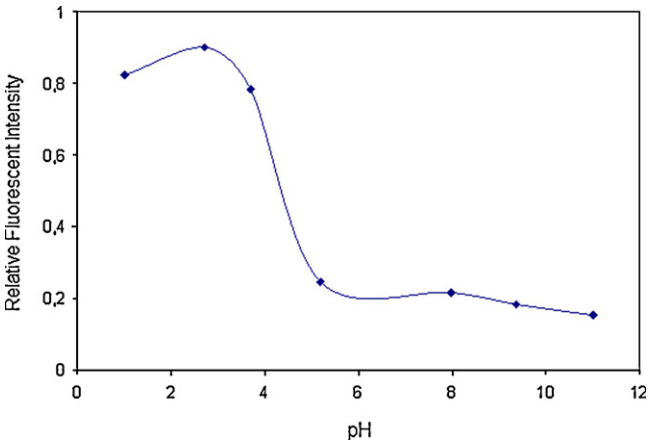

Fig. 5. Effect of pH on FUR (0.16 μg mL^{-1}) fluorescence intensity in solution. Slit width ex: 5 nm, em: 10 nm.

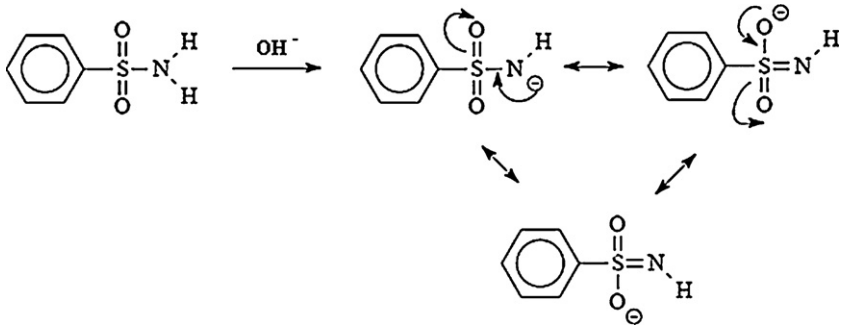

Fig. 6. Delocalization of the negative charge upon the oxygens of molecules containing sulfonamide groups by resonance.

Table 1
Validation of the method for AMI determination in commercial pharmaceutical formulae.

Samples	Base value ($\mu g\,mL^{-1}$)	Added ($\mu g\,mL^{-1}$)	Found[a] ($\mu g\,mL^{-1}$)	Recovery[b] %	RSD %
A	0.100	0.100	0.198	98.0	1.11
B	0.100	0.200	0.295	97.5	0.71
C	0.100	0.300	0.398	99.3	0.44
D	0.100	0.400	0.514	103.5	1.36

[a] Mean value, $n = 6$.
[b] $\times 100$ [(found − base)/added].

Table 2
Validation of the method for FUR determination in commercial pharmaceutical formulae.

Samples	Base value ($\mu g\,mL^{-1}$)	Added ($\mu g\,mL^{-1}$)	Found[a] ($\mu g\,mL^{-1}$)	Recovery[b] %	RSD %
A	0.400	0.400	0.823	105.7	0.92
B	0.400	0.800	1.235	104.3	1.28
C	0.400	1.200	1.627	102.2	1.23
D	0.400	1.600	2.100	106.2	0.77

[a] Mean value, $n = 6$.
[b] $\times 100$ [(found − base)/added].

time involved in this step was 2 min 30 s for each sample/standard, achieving that the whole analysis process be very rapid.

3.4. Fluorescence characteristics of FUR in aqueous media

Fig. 4 shows the excitation and emission spectra obtained for a aqueous solution of FUR 0.16 $\mu g\,mL^{-1}$ at pH 2.7. As can be seen, the drug presents an emission maximum at 415 nm when is excited at 237 nm. These wavelengths are selected to measure the fluorescence intensity in the following assays. In this figure can also be observed, the spectral interference between AMI and FUR.

3.5. Effect of pH on FUR separation and fluorescence intensity

Fig. 5 shows the effect of pH value on the FUR fluorescence signal in aqueous solution. The spectrum presents maximal intensity between pH 2 and 3, and decreases significantly at pH higher than 4. The working pH selected was 2.7.

Table 3
Analytical figures of merit.

Parameter	Amiloride	Furosemide
Linearity range ($\mu g\,mL^{-1}$)	3.20×10^{-4} to 0.8	1.33×10^{-3} to 4
LOD[a] ($\mu g\,mL^{-1}$)	9.62×10^{-5}	4.01×10^{-4}
LOQ[b] ($\mu g\,mL^{-1}$)	3.20×10^{-4}	1.33×10^{-3}
Slope (m)	1091.2	156.9
Intercept (h)	198.68	78.587
r^2	0.9835	0.9957

r: correlation coefficient.
[a] 3σ criterion.
[b] 10σ criterion.

The acidity constants values for FUR are: $pK_{a1} = 3.70$, $pK_{a2} = 10.31$ [22]. The pK_{a1} for FUR corresponds to the ionization of carboxylic group; the pK_{a2} value corresponds to the ionization of sulfonamide group [23]. At pHs above 5, FUR is predominantly in its negatively charged form and it is not significantly retained by the non-polar nylon membrane. The maximum fluorescence intensity of FUR at pH 2.7 could be explained by the presence of a carboxylic group totally undissociated, which can form intermolecular hydrogen bonds with the carboxylic groups of other FUR molecules. This bond, which involves part of the fluorophore, gives an additional rigidity to FUR molecule producing an enhancement in the fluorescence emission.

Thus, the problem of the existence of overlapping in fluorescence emission spectra of AMI and FUR in aqueous solutions, could be solved separating the analytes through a polyamide membrane. The optimum separation conditions were obtained filtering at pH 11, at which AMI keeps as neutral specie retained by the polyamide membrane, while the anionic form of FUR, remains in solution. Both drugs can be spectrofluorimetrically determined, on the membrane and in the filtered solution, respectively.

3.6. Characteristics of polyamide membranes

The unalterable surface of polyamide membranes respect to the drastic pH changes during the experiments and the obtained low fluorescent background when the organic compound extracted on it is spectrofluorimetrically measured, demonstrate that this membrane is advantageous and appropriate to the proposed objective.

Nylon membranes of different batches and marks were tested, all of them of 0.45 μm pore size: Millipore (USA) and Gamafil (Argentine Industry). There was not observed significant differ-

Table 4
Analysis of AMI and FUR in pharmaceutical samples.

Samples	AMI nominal (mg)	AMI found[a] (mg)	Recovery[b] %	RSD[c] %	FUR nominal (mg)	FUR found[a] (mg)	Recovery[b] %	RSD[c] %
1	5.00	5.15	103	0.71	40.00	40.45	101.1	0.52
2	5.00	5.20	104	0.43	40.00	41.00	102.5	0.67
3	5.00	5.06	101	1.42	40.00	39.00	97.5	1.24

1: Nuribán-A® (Lab. Roux-Ocefa, Bs. As., Argentina) (tablets). 2: Errolón-A® (Lab. Sandoz, Bs. As., Argentina) (tablets). 3: Lasiride® (Lab. Aventis, Bs. As., Argentina) (tablets).
[a] Mean value, $n = 6$.
[b] Recoveries were calculated considering the drugs contents labeled by the manufacturing laboratories.
[c] Relative standard deviation.

ences from these membranes, neither in background emission nor in the luminescence properties of the retained AMI. None of these membranes required special conditioning and were used as-received.

3.6.1. Polyamide membranes performance in solid-phase extraction

Nylon is a naturally hydrophilic membrane, compatible with both, aqueous and organic solvents [24]. AMI and nylon have in their structures, tertiary and secondary amide groups, respectively, and thus a possible explanation for the observed phenomenon of chemisorption is that the analyte interacts with the support by forming hydrogen bonds with it. Similarly, it could also be explained the fact that at pH 11, FUR is not retained by this membrane. At this pH the carboxylic group in FUR is totally dissociated, stabilized by resonance and the negative charge upon the oxygen is delocalized, diminishing the possibility of forming a hydrogen bond. FUR sulfonamide group acts as a weak acid. This acidic nature results from the ability of the SO_2 moiety to stabilize the nitrogen anion through resonance, delocalizing the negative charge upon the oxygens (Fig. 6). This behaviour, favoured in alkaline media, produces the lack of interaction between the membrane and FUR.

Savvin et al. [25,26] studied the chemofiltration processes and suggested that the retention mechanism for organic reagent complexes on the polyamide membrane filter is based not only on electrostatic forces but also on non-hydrophobic interactions between the polymeric matrix of the membrane and the molecules of organic reagent. In our research group, the chemofiltration processes have already been successfully applied with other purposes: the preconcentration and determination of trace rare earths. The formation and retention by chemofiltration of different metal complexes were studied, obtaining high grade of recovery [27–29].

Some researchers have reported the determination of pharmaceuticals using the nylon membranes only as support, unloading little volumes of sample on them [24] and, then, the solvent is evaporated, before measuring the emission. Other reported method for concentrating organic drugs [30] using nylon membranes was realized forcing the sample solution to pass through a restricted area of the membrane surface, taking up to 30 min, in this step, with each sample or standard. The separation of organic drugs through a chemofiltration process has not yet been reported.

3.6.2. Evaluation of the separation-preconcentration methodology

In order to confirm the complete extraction of AMI on a single disc, a second disc was placed in the filtration holder, below the first nylon membrane, and then the fluorescence signal in this second support was measured. This procedure was repeated using solutions containing different concentrations of AMI. In all studied cases, the spectra were equal to those obtained with the blank, suggesting that complete retention of AMI in the first nylon disc was achieved. Although the nylon membrane is very thin and its extraction capacity is likely to be limited, it does not seem to be exceeded at the AMI concentration levels employed.

The enrichment factor is defined as: $F = (Q_T/Q_M)/(Q_T^0/Q_M^0)$, where Q_T^0, Q_T are the analyte quantities before and after the preconcentration, and Q_M^0, Q_M are the amounts of the matrix before and after the enrichment [31]. Considering a recovery percentage of almost 100%, an initial volume of 10 mL, a final volume consisting in a film of 4 μm thickness and a filtration surface of 17.35 cm^2 (membrane surface area), the enrichment factor is 1500. The achieved preconcentration combined with the enhancement in fluorescence signal by the immobilization gives a highly sensitive method for AMI determination.

3.7. Precision and accuracy: validation

The precision of the method based on repeatability was determined, by replicating the method ($n = 6$) on four sample solutions of the same batch of commercial tablets of AMI/FUR using the standard addition method, giving a relative standard deviation minor than 1.23 for FUR and 1.36 for AMI. The validation method results are shown in Tables 1 and 2.

3.8. Determination of AMI and FUR in pharmaceuticals

AMI and FUR can be easily separated and determined in commercial formulations using the method herein described. The spectrofluorometric determination of AMI on nylon membrane and FUR in the filtered solution, involved the construction of the corresponding calibration curves. The equation for the calibration graphs is: $F = h + mC$, where F is the fluorescence intensity and C is the concentration of AMI or FUR. The figures of merit obtained are displayed in Table 3, which demonstrate the good performance of the calibration.

The commercial pharmaceutical samples analyzed are presented in Table 4, and the values obtained indicate that the applied method yields satisfactory results in all studied cases. The spectra of AMI and FUR in pharmaceutical samples separated by the proposed methodology compared to those obtained with the standard solutions, showed no changes, confirming the absence of interferences from excipients. In addition, the spectrum of the membrane alone and the spectrum of a synthetic solution containing only the excipients of the formula (carboxymethyl cellulose 2%, starch 10%, magnesium stearate 0.8%, gelatin 4%, lactose 83.2%) filtered on the membrane, did not show fluorescence signals, showing that they are not adsorbed on the membrane.

4. Conclusions

The present study demonstrates the feasibility of using a membrane as a novel support for solid-phase extraction procedures, focused on AMI and FUR separation and determination. This nylon property is an important point to be considered when water samples are filtered via nylon discs before chromatographic determinations of AMI, especially if low concentrations are analyzed.

The high preconcentration factor obtained with the chemofiltration through the membrane, associated with the sensitivity

of molecular fluorescence techniques and the simplicity of the applied experimental procedure, provide an outstanding method for AMI determination. The different behaviour of both studied drugs respect to the membrane, constitutes an economic and simple separation methodology that permits to resolve spectral interferences and determine AMI and FUR with high sensitivity and precision.

An additional advantage, which should also be considered, is the avoidance of using toxic organic solvents, which are usually involved in more conventional extractions and elution procedures.

Compared to other supporting materials, nylon has the following advantages: (1) it is easily available and cheap and (2) it does not require pre-treatments. Therefore, the use of nylon considerably reduces the analysis costs for numerous samples.

Taking into account the very satisfactory limits of detection obtained from fluorescence measurements, and also the fact that auxiliary reagents are not necessary for this latter technique, it can be assert that the proposed separation and determination methodology constitutes a good alternative to chromatographic ones. According to our results, this capability of nylon could be extended to the analyses of other drugs.

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