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Solid phase extraction using nylon membranes with fluorescence detection as a fast and sensitive method for Amiloride and Furosemide determination in urine samples

Cecilia M. Peralta [a,*], Liliana P. Fernández [a,b], Adriana N. Masi [a,b]

[a] Instituto de Química de San Luis (INQUISAL-CONICET), Argentina
[b] Área de Química Analítica, Facultad de Química, Bioquímica y Farmacia, Chacabuco y Pedernera, Universidad Nacional de San Luis, 5700 San Luis, Argentina

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ABSTRACT

A diuretic is a substance widely prohibited in competition sports, by the World Anti-Doping Agency (WADA). In this paper, a sensitive, rapid and convenient analytical solid phase extraction-fluorimetric method for the determination of furosemide (FUR) and amiloride hydrochloride (AMI) diuretics in human urine was developed. FUR and AMI exhibit overlapped fluorescent spectra and urine produces background fluorescence that precludes the direct determination of these diuretics by conventional fluorimetry. AMI was adsorbed using nylon membranes whereas; FUR was detected in aqueous solutions. The optimum analytical conditions for AMI and FUR assay were established. Excitation wavelengths of 365 nm and 237 nm and emission wavelengths of 406 and 415 nm were used for AMI and FUR, respectively. The calibration graphs are linear in the range of 3.7×10^{-4} to 0.8 μg mL^{-1} and 1.2×10^{-3} to 4.0 μg mL^{-1}, for AMI and FUR, respectively, with a detection limit of 1.1×10^{-4} and 3.5×10^{-4} μg mL^{-1} (S/N = 3). These concentrations correspond well with the range of the drugs in urine. AMI and FUR were accurately quantified in doped urine samples and urine of subjects under medical treatment with diuretic. The results were validated by recovery test, being highly satisfactory.

1. Introduction

Anti-doping analysis is a very peculiar area of forensic toxicology, aimed at detecting the abuse of prohibited substances and methods by the athletes. The organization of the analytical work requires detailed planning of all the experimental, logistic and administrative activities in order to set up a "laboratory system" capable of detecting the illicit recourse for prohibited substances and methods, with response times that can be as short as 24 h from the reception of the samples in the case of major international sport events [1].

In most cases, team doctors administer doping substances to athletes with the aim of improving their performance. The recent growth in the use of doping substances, masking agents that hinder the excretion of such prohibited substances and the uncontrolled use of medical drugs has led competent bodies to promote the development of analytical methods for these substances in urine. For such methods to be effective, they should be direct and selective, and possess low limits of detection.

A great portion of the analytical methods set up and followed in the anti-doping laboratories rely on mass spectrometric techniques, and especially on the combination between chromatography (both gas and liquid chromatography) and mass spectrometry (MS).

Diuretics are substances that produce increase urinary excretion of bodily sodium bound to anions and water, thereby reducing its concentration in extracellular liquids. AMI (*N*-amidino-3,5-diamino-6-chloropyrazine-2-carboxamide) and FUR (4-chloro-N-furfuryl-5-sulfamoylanthranilic acid), the chemical structures of which are shown in Fig. 1, are two natriuretic agents used to treat various types of disease associated to sodium high level [2].

FUR is a loop diuretic, used in the treatment of congestive heart failure and edema. FUR works by blocking the absorption of salt and fluid in the kidney tubules, causing a profound increase in urine output (diuresis). The diuretic effect of FUR can cause body water and electrolyte depletion. Therefore, careful medical supervision is necessary during treatment [3].

AMI has the property of interfering with the process of cationic interchange in the distal tube [4]. It blocks the absorption of sodium ions and the excretion of potassium ions.

The association of FUR and AMI furnishes a valuable natriuretic agent with a diminished kaliuretic effect, minimizing the risk of alkalosis in the treatment of refractory oedema associated with hepatic cirrhosis or congestive heart failure [5].

AMI and FUR have been included, by the International Olympic Committee, in the list of forbidden drugs [6]. The prohibition is principally due to two factors: (1) the drugs allows for a rapid decrease of the corporal weight (an important factor in sports where weight categories are involved) and (2) it conceals the ingestion of other proscribed agents, because the profuse amount of eliminated

* Corresponding author. Tel.: +54 2652425385; fax: +54 2652430224.
E-mail address: cperalta@unsl.edu.ar (C.M. Peralta).

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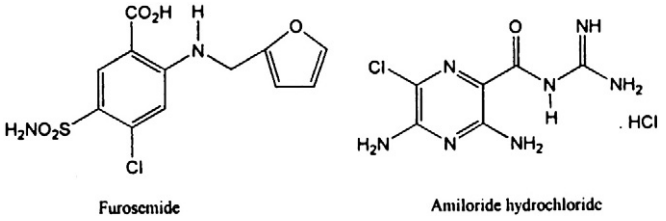

Fig. 1. Chemical structures of furosemide and amiloride hydrochloride.

liquid reduces their concentration in urine [7]. No medical reason can justify a rapid loss of weight in any sport; in fact, abusing the drugs used for this purpose can pose serious health risks through adverse secondary effects [2].

The therapeutic and doping dose of AMI varies from 5 to 20 mg daily (one administration only). It is incompletely absorbed and it does not appear to be metabolized. The half-life in plasma varies from 6 to 10 h and about 50% of an oral dose is excreted in the unchanged form in urine [8].

AMI exhibits an extremely low therapeutic range (0.5–25 ng ml^{-1}), and therefore only a few sufficiently specific and sensitive HPLC methods have been described [9–14].

A characteristic of AMI is that low doses lead to high-volume urine excretion, making its determination difficult owing to the low concentrations and therefore highly sensitive methods are required [15].

FUR is administered both orally and intravenously. The pharmacokinetics of FUR is well documented in healthy subjects. Absorption is rapid and peak levels occur after 60–90 min post-dose. It has a high plasma protein binding (97–98%) and is eliminated by hepatic and renal glucuronidation and by renal secretion and filtration [16]. The elimination half-life is relatively fast ($t_{0.5}$ 0.5–2 h).

Several HPLC, HPLC/MS and CE methodologies have been developed for the determination of FUR in biological fluids [17–24]. However, employing classical HPLC columns can be rather time consuming due to pretreatment samples.

This paper reports a method for the sequential determination of both diuretics in urine, using a novel separation method through membrane immobilization prior emission fluorescence detection.

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 filtration with in line Millipore XX4304700 (USA) polypropylene filter holder of 47 mm i.d, closing nylon butterflies and silicon toric joint. Solutions were propelled through the filter holder with 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.

A centrifuge (Rolco® Argentine Industry) was used in biological samples processing.

2.2. Reagents

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

The pH values in optimization stage were adjusted by the addition of solutions of 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 target pH value was reached.

2.2.1. Standard solutions

Standard solutions 10 μg mL^{-1} of AMI and FUR (Lab. Puntanos, San Luis, Argentina) were prepared by dissolving 5 mg of each compound in 500 mL of ultrapure water. In these conditions, both solutions were found to be stable for at least 2 months when kept in dark. Standard working solutions were prepared daily by appropriate dilution using the same solvent.

2.2.2. Human urine samples

Fresh human urines, obtained from healthy volunteers, were collected and homogenized. Similarly, urine from one patient under Errolón–A® (contained 5 mg AMI and 40 mg FUR) treatment was collected immediately before its administration and at intervals between 0 and 2, 2 and 4, 4 and 6, and 6 and 12 h after drugs intake.

Aliquots of 10 mL were placed in graduated centrifuge tubes. These solutions were then centrifuged for 10 min at 3500 rpm ($1350 \times g$) and 3 mL of supernatants were transferred into new test tubes and stored at 4 °C until assays.

2.2.3. Sample preparation

Aliquots of 200 μL centrifuged human urine samples were doped with different concentrations of AMI and FUR (0.25–2 μg of AMI and 2–16 μg of FUR). The spiked samples were put in 10 mL centrifugal tubes and were processed as described in general analytical procedure.

2.3. General analytical procedure

2.3.1. Solid phase extraction (SPE)—nylon surface fluorescence

Nylon membranes, without previous conditioning, were placed in Millipore filter holder and 10 mL portions of urine sample or standard solution 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 for FUR determination. After the membrane was dried, the disk was placed in a solid sample holder, and the fluorescence spectrum was scanned at 90°. For the AMI determination, 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. Fluorescence determination in filtered solution

For the FUR determination, the filtered solutions obtained from Section 2.3.1 procedure were conditioned to pH 2.7 by adding HCl solution. Then sample and standards solutions were introduced into 1 cm path length cell in the spectrofluorometer and the fluorescent emission was measured at $\lambda_{em} = 415$ nm using $\lambda_{ex} = 237$ nm. In all cases the excitation and emission slit widths were of 5 and 5 nm, respectively.

3. Results and discussion

3.1. Fluorescence characteristics

As can be seen in Fig. 2, AMI exhibits two excitation bands at λ_{ex1} 285 and λ_{ex2} 365 nm, with emission at λ_{em} 424 nm. These bands are virtually identical in intensity. FUR also exhibits excitation bands at λ_{ex1} 237, λ_{ex2} 275 nm and λ_{ex3} 340, with emission at λ_{em} 410 nm. In this figure can be observed, the spectral interference existing between AMI and FUR.

Urine introduces additional complications as it contains a variety of organic substances. Most such substances exhibit a high absorbance

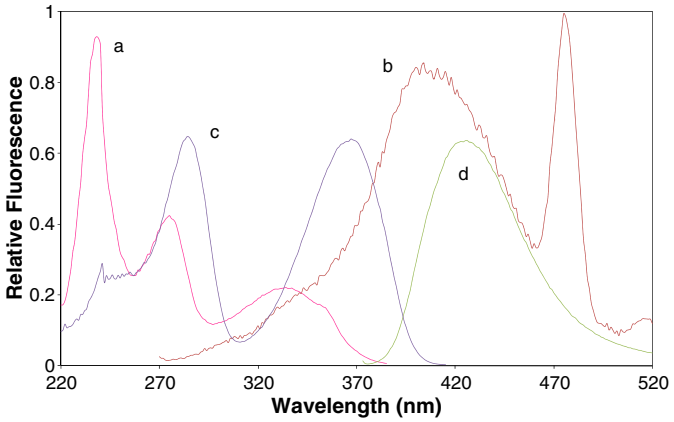

Fig. 2. Excitation and emission spectra of FUR and AMI. Slit width ex: 5 nm, em: 10 nm. FUR (0.16 μg mL^{-1}, pH 2.7) (a) excitation spectrum (λ_{em} = 415 nm) (b) emission spectrum (λ_{ex} = 237 nm). AMI (0.16 μg mL^{-1}, pH 11) (c) excitation spectrum (λ_{em} = 424 nm) (d) emission spectrum (λ_{ex} = 365 nm).

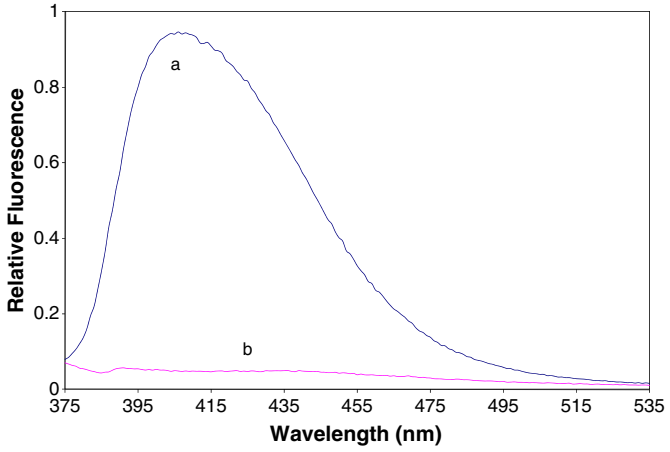

Fig. 4. Emission spectrum of (a) AMI (0.8 μg mL^{-1}) and (b) urine (λ_{ex} = 365 nm and λ_{em} = 406) retained on membranes. Slit width 1.5 nm.

in the ultraviolet (UV) region but only a few exhibit fluorescence [25]. On the other hand, the fluorescent components of urine exhibit strong background fluorescence that interferes with the direct determination of a great number of analytes.

Fig. 3 shows the emission spectra for FUR and AMI solutions at their optimal pH and excitation conditions, and the emission spectra of urine realized at the same conditions of each drug. The fluorescent spectrum exhibited by urine at AMI excitation wavelength presents an important overlapping with the AMI emission spectrum. Meanwhile, at optimal pH and excitation conditions of FUR, urine does not present fluorescent emission that interferes with the emission of FUR.

The emission fluorescent spectrum obtained for AMI supported on a solid substrate can be seen in Fig. 4. It is also important to observe the absence of fluorescence signals in the blank spectrum. This blank was prepared by filtrating urine without addition of AMI with the polyamide membrane. It can be concluded that the urine compounds are not retained by the membrane, demonstrating the efficacy of this separation method for eliminating interference from urine, which has a highly fluorescent matrix.

3.2. Influence of experimental variables

In order to choose the optimal experimental conditions for AMI and FUR separation, our previous work about the determination of AMI and FUR in pharmaceuticals [26], was considered. In spite of the different characteristics of the samples, these conditions were not modified when working with the urine matrix.

As a result, for separation and determination of AMI, pH 11 (adjusted with NaOH) was selected. AMI molecule must stay in its neutral form for achieving quantitative retention on the non-polar polyamide membrane. Thus, in basic solution, hydroxyl ions may react with AMI to produce neutral species, which are retained on the membrane. As previously studied, at pHs above 5, FUR is predominantly in its negatively charged form and it is not significantly retained by the used non-polar nylon membrane.

The maximum fluorescence intensity achieved for FUR at pH 2.7 (adjusted with HCl) 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.

Based on selectivity, sensitivity, and reproducibility criteria, the bands of characteristic wavelengths λ_{ex} = 365, λ_{em} = 406 and λ_{ex} = 237, λ_{em} = 415 were selected to determine AMI and FUR, respectively. A flow rate of filtration of 4 mL min^{-1} was selected as optimal.

The problem of the existence of overlapping in fluorescence emission spectra of AMI and FUR in urine solutions could be solved by separating the analytes through a polyamide membrane by chemofiltration at AMI retention pH, while the anionic form of FUR, remains in solution. 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. In, addition, the preconcentration produced by the chemofiltration process gives a beneficial enhancement of the fluorescence signal. It is evident that the separation process on the polyamide membrane, not only separates FUR from AMI but also the urine compounds which overlap AMI emission spectra and which do not emit fluorescence at FUR determination conditions.

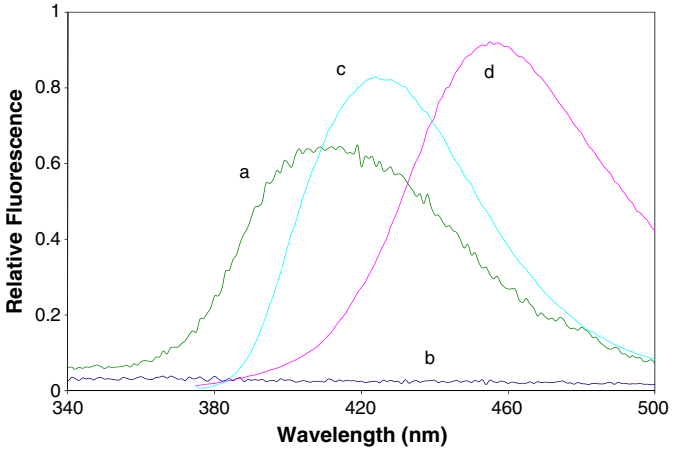

Fig. 3. Emission spectra of FUR, AMI and urine. Slit width 5 nm. (a) FUR (3.2 μg mL^{-1}), (b) urine 1/50 at pH 2.7 (λ_{ex} = 237), (c) AMI (0.8 μg mL^{-1}) and (d) urine 1/50 at pH 11 (λ_{ex} = 365).

Table 1
Analytical figures of merit.

Parameter	Amiloride	Furosemide
Linearity range (μg mL^{-1})	3.7×10^{-4}–0.8	1.2×10^{-3}–4
LOD[a] (μg mL^{-1})	1.1×10^{-4}	3.5×10^{-4}
LOQ[b] (μg mL^{-1})	3.7×10^{-4}	1.2×10^{-3}
Slope (m)	1894.1	253.82
Intercept (h)	40.143	35.82
r^2	0.9988	0.9944

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

Table 2
Recovery test of AMI in spiked human urine.

Aliquots[a]	AMI added ($\mu g\ mL^{-1}$)	AMI found ($\mu g\ mL^{-1}$)	Recovery (%)[b] Intraday	RSD (%)[b] Intraday	AMI found ($\mu g\ mL^{-1}$)	Recovery (%) Interday[c]	RSD (%) Interday
I	0.025	0.0254	101.6	3.76	0.0261	104.4	7.30
II	0.050	0.0510	102.0	2.46	0.0510	102.0	4.76
III	0.100	0.1073	107.3	2.05	0.1100	110.0	5.71
IV	0.150	0.1517	101.1	1.49	0.1428	95.2	2.90
V	0.200	0.2003	100.1	1.41	0.2109	105.4	2.49

λ_{ex} 365 nm, λ_{em} 406 nm; slit width ex: 1.5 nm, em: 1.5 nm.
[a] Aliquot of 200 μL of urine sample.
[b] Average of six determinations.
[c] Three days.

Table 3
Recovery test of FUR in spiked human urine.

Aliquots[a]	FUR added ($\mu g\ mL^{-1}$)	FUR found ($\mu g\ mL^{-1}$)	Recovery (%)[b] Intraday	RSD (%)[b] Intraday	FUR found ($\mu g\ mL^{-1}$)	Recovery (%) Interday[c]	RSD (%) Interday
I	0.2	0.219	109.5	5.21	0.211	105.5	7.90
II	0.4	0.402	100.5	3.93	0.429	107.2	5.57
III	0.8	0.867	108.3	3.89	0.732	91.5	5.01
IV	1.2	1.191	99.2	2.54	1.268	105.6	3.90
V	1.6	1.581	98.8	1.56	1.666	104.1	3.50

λ_{ex} 237 nm, λ_{em} 415 nm; slit width ex: 5 nm, em: 5 nm.
[a] Aliquot of 200 μL of urine sample.
[b] Average of six determinations.
[c] Three days.

3.3. Polyamide membrane as extractive support

As demonstrated in previous works, nylon proved to be an adequate solid-support for detecting fluorescence and phosphorescence luminescence emission of certain organic compounds deposited on it [27–30]. The latter studies were performed using the classical procedure which involves only the deposit of the studied solution on the nylon surface. However, a clear improvement in the limits of detection is noted when a solid phase extraction procedure is carried out [30,31]. Our research group has experience in using nylon membranes for the chemofiltration of chelating agents and has successfully applied them with different purposes [32–34].

The main advantage of using nylon membranes as support for spectrofluorimetry lies in the absence of fluorescence emission in the spectral region of interest. It means that at the emission wavelength of AMI, the support has a very low spectral background. In addition, the filtration processes generally sweep some components from the filter matrix which, in this case, do not interfere with the following determination of FUR.

It is important to remark that the direct measurement of the analyte on the solid surface avoids the drawbacks associated to compounds desorption needed for the analysis in solution, such as the increase in the percentage of error in final data [31].

Other advantage of the described procedure for analysis of AMI and FUR in urine is the simplicity of the sample pretreatment, since it is avoided the hazardous successive extraction–washing steps or the need of employing a high performance separation instrumental for the treatment of urine previous measurement, which in most cases is necessary for eliminating interferent species.

3.4. Analytical figures of merit

The spectrofluorometric determination of AMI on nylon membrane and FUR in the filtrated 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 the concentration of AMI or FUR. The figures of merit obtained are displayed in Table 1, which demonstrate the good performance of the calibration.

Sample preconcentration by SPE provides AMI the levels of detection needed for urine analysis. According to the LOD obtained with the chemofiltration on nylon membranes (Table 1), the detection of AMI in urine samples (e.g. 0.1 $\mu g\ mL^{-1}$) is possible. Simultaneously, the enhancement in FUR fluorescence emission at working conditions allows its determination in the low concentrations found in urine samples.

3.5. Validation

3.5.1. Determination of AMI and FUR in urine samples

AMI and FUR can be easily separated and determined in urine using the method herein described. The precision intraday and interday of the method based on repeatability was performed, by replicating the separation and determination process ($n = 6$) on five spiked urine samples using the standard addition method and covering different concentration levels. The spiked concentrations varied between 0.025 and 0.2 $\mu g\ mL^{-1}$ for AMI and 0.2 to 1.6 for FUR according to the estimated value of these drugs in actual samples from patients under treatment. The relative standard deviation was minor than 7.9% in all cases. The intraday and interday recovery ranged from 91.5 to 109.5%. Validation results are shown in Tables 2 and 3. In order to evaluate the feasibility of applying the developed methodology to urine samples of patients under diuretic treatment, recovery test was carried out. Table 4 shows the obtained results for FUR and AMI contents in urine after single dose of this formulation.

Table 4
Analysis of AMI and FUR in urine sample of patient under diuretic treatment ($n = 6$).

Drug	C_{max} found ± SD ($\mu g\ mL^{-1}$)	C_{min} found ± SD ($\mu g\ mL^{-1}$)	C_{mean} found ± SD ($\mu g\ mL^{-1}$)
Amiloride	0.0735 ± 0.008	0.0439 ± 0.009	0.0581 ± 0.004
Furosemide	0.4810 ± 0.005	0.1885 ± 0.007	0.2947 ± 0.006

C_{max} is the maximum concentration of drug.
C_{min} is the minimal concentration of drug.
C_{mean} is the medium concentration of drug.
SD is the standard deviation.

4. Conclusions

These are probably two essential reasons for the growing use of fluorimetry in pharmaceutical analysis. In fact, this technique is the most widely favoured instrumental method for detecting small amounts of therapeutic and abuse drugs in biological materials, where they frequently occur at levels as low as a few nanograms per millilitre. The added economy and simplicity, and the relative flexibility of fluorimetry, have turned it into the officially recommended choice for determining the purity of many drugs or their contents, for example, in pharmaceutical, forensic and biomedical samples [35].

The new method for the sequential determination of AMI and FUR in urine by fluorimetry proposed here is simple, efficient and allows resolving the mixture by using a solid phase extraction to suppress the background fluorescence of urine and avoid the overlap between the AMI and FUR spectra. The use of a solid-support, combined with the use of fluorimetry technique, contributes to enhancing both sensitivity and selectivity, so performing simultaneously the separation (retention), preconcentration and detection of the analytes.

In conclusion, the method described in the current paper fulfills the precision, linearity, sensitivity, and specificity requirements to quantify AMI and FUR in doped urine samples and urine of subjects under medical treatment with diuretic. Furthermore, the procedure appears to be cheaper than those previously described. It has the additional advantage to avoid the use of toxic organic solvents, which are usually involved in more conventional extraction and elution procedures. Altogether these factors make this method a useful tool to be used for monitoring of AMI and FUR levels in small volumes of urine samples.

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