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Automated flow system for sildenafil enrichment using surfactant coated solid-phase with fluorescence detection

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"In memoriam of Dr. Adriana Masi, prominent researcher, dear colleague and a friend who passed away prematurely as a consequence of public insecurity killed by a shot in the head at the door of her house"

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ABSTRACT

In this work, Amberlite XAD-1180 resin is used for on-line surfactant-mediated pre-concentration of sildenafil as a prior step for its fluorescent detection. In order to activate the column for sildenafil pre-concentration, the cationic surfactant (hexadecyltrimethylammoniunm bromide, HTAB) is adsorbed onto the resin. In these conditions, sildenafil is retained by HTAB–resin and then it is eluted with ethanol and analyzed by spectrofluorimetry. Drug–surfactant association produces a considerable fluorescence enhancement, increasing considerably the sensitivity of detection. Therefore, sildenafil can be pre-concentrated and quantitatively determined, with a detection limit of 0.2 ng mL^{-1}. The proposed method was successfully applied to the analysis of bulk drug, human urine, tablets, and local herbal medicine. Validation processes were performed by recovering studies and statistical analysis with satisfactory results.

1. Introduction

Sildenafil (widely known as Viagra) is a selective inhibitor of cyclic guanosine monophosphate specific phosphodiesterase type 5. Clinically, it is an effective drug for the treatment of erectile dysfunction (ED). It is also used for pulmonary hypertension, Raynaud's phenomenon, altitude sickness and Duchenne/Becker muscular dystrophy [1]. As a selective pulmonary vasodilator, sildenafil improves gas exchange, increasing the life expectancy and exercise tolerance which has encouraged its use in premature infants with severe respiratory failure, children with primary and post-surgical pulmonary hypertension and severe lung fibrosis [2].

Sildenafil interacts with some medicine (e.g., nitroglycerine, doxazosin and terazosin) used for hypertension and ischemic heart disease treatment [3]. Moreover, since sildenafil is promoted for the treatment of ED, it is one of the most widespread drugs of use and abuse. Because of its popularity, it has been reported that there is an illicit addition of this drug in a wide variety of products such as herbal medicine for ED and dietary supplements. Therefore, its determination in non-official formulations such as herbal medicines and dietary supplements has raised concerns by national and international health authorities around the world [4].

The pharmacokinetic profile of sildenafil has already been well established including the correlation of concentration between plasma and urine [5,6]. A single oral dose (20 mg of sildenafil) administered to 10 healthy men yielded an average concentration in plasma of 50 μg L^{-1} at 1.5 h [7]. The average value for bioavailability of sildenafil is 41% with elimination half-lives of 4 h, largely as products of biotransformation in feces (80%); only 13% is excreted by urine [8]. In different reported post-mortem studies, sildenafil concentrations of 40–105 μg L^{-1} were found in blood, and 63–246 μg L^{-1} in urine [9–11].

In clinical analysis, urine collection is a simple and non-invasive sampling method, in contrast to blood sampling which is an invasive procedure and requires trained personnel. Moreover, blood extraction frequently produces stress and annoyance for patients.

Several methods have been developed for sildenafil determination [12], such as spectrophotometric [13,14], chromatographic [7,15–18], MEKC [19], voltammetric [20–22], potentiometric [23], ^{1}H-NMR [24], and spectrofluorimetric [25] methods. Even though UV–visible is the routine detection method used, in previous work we have demonstrated that on using fluorescence detection

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mediated by surfactants an improvement in sensitivity is achieved. In addition, surfactants are less harmful to the environment and human health than traditional organic solvents [26].

Due to the complex matrix involved in biological fluids and the wide variety of non-registered formulations, the determination of sildenafil is generally a difficult task. Furthermore, the low concentration of the drug normally found in these samples makes necessary the use of separation and pre-concentration techniques, which are time-consuming involving tedious operational steps.

To reduce the sampling rate, an automated system involving on-line sample pretreatment is preferred [27–29]. Flow injection analysis (FI) is a well-established on-line technique with numerous and widespread applications in quantitative chemical analysis. Compared to batch methods, FI offers an increased sampling rate, lower reagents consumption, better precision and high versatility. These advantages have led to a continuously increasing interest for pharmaceutical analysis and quality control applications [30].

Amberlite XAD resins are extensively used for solid-phase extraction techniques. They are highly porous spherical polymer beads based on cross-linked, macro-reticular polystyrene, aliphatic, or phenol–formaldehyde condensate polymers. Their high internal surface area can adsorb and desorb a wide variety of organic compounds and inorganic species [31–33]. The increasing interest on XAD resins is due to their effective separation from very dilute aqueous solutions, favorable elution and regeneration characteristics, high selectivity, low toxicity and relatively inexpensive costs. Hence, XAD resins are used for bulk separation and purification in food, pharmaceuticals and chemical industries [34].

In this paper, different adsorbent resins have been studied in order to pre-concentrate sildenafil prior to its determination. FIA manifold has been designed for its on-line analysis and the variables have been studied. This proposed method has been validated and applied for sildenafil determination in urine sample and herbal medicine extracts for ED treatment.

2. Experimental

2.1. Instrumentals

A Shimadzu RF-5301PC spectrofluorimeter (Shimadzu Corporation, Analytical Instrument Division, Kyoto, Japan), equipped with a Xenon discharge lamp and a quartz flow-through cell was used for the fluorescent measurements.

Solutions were propelled by Gilson Minipuls 3 peristaltic pump with PVC pumping tubes and two valves were used for FIA configuration. Valve 1 was a 3-channel 1-way valve (Rheodyne, Model 5041) and valve 2 was a homemade 12-channel 6-ways valve (kindly provided by Prof. B.F. Reis, Sao Paulo University, Brazil).

A pH meter (Orion Expandable Ion Analyzer, Orion Research, Cambridge, MA, USA) Model EA940 with combined glass electrode was used for monitoring pH adjustment.

2.2. Reagents and samples

Sildenafil (as citrate) was kindly provided by Gador S.A. (Buenos Aires, Argentina).

Amberlite XAD resins were purchased from Rhom-Haas (Philadelphia, USA). All XAD resins were previously activated with HNO_3(c)/ethanol (1:4) for about 4 h.

Reagents of analytical grade were used. HTAB and SDS (sodium dodecylsulfate) were purchased from Tokyo Kasei Industries (Chuo-Ku, Tokyo, Japan). NaOH and HCl were purchased from Merck (Darmstadt, Germany).

Ethanol (HPLC grade) was used as the eluent.

2.2.1. Assay solutions

Sildenafil standard solution containing 2.0 mg mL^{-1} was prepared by dissolving the reagent with bi-distilled water and conditioned to pH 11 with NaOH(d). This solution was stable for at least 2 weeks stored at room temperature.

HTAB solution (1.0×10^{-3} mol L^{-1}) was prepared using an adequate weight of the reagent and dissolving in bi-distilled water.

2.2.2. Samples pretreatments

Human urine. Fresh matinal human urine was obtained from healthy volunteers. After centrifugation, supernatants were collected and stored in a sterile container at ~5 °C for further use.

Herbal medicines. Aqueous extract of *Lycopodium saururus* was obtained by infusion of 5 g (dry weight) of the commercially available medicine with 80 mL of boiling ultra pure water. After 5 min., the mixture was filtered and made to 100 mL with ultra pure water.

Aqueous extract of a herbal mixture (*Haploppapus baylahuen*, *L. saururus*, *Baccharis articulata*, *Thymus vulgaris* and *Salvia apiana*) was obtained by infusion of 5.0 g (dry weight) of this commercially available mixture with 80 mL of boiled ultra pure water (approximately 100 °C). After 5 min, the mixture was filtered and made up to 100 mL with ultra pure water.

Pharmaceutical formulation (tablets). Five tablets of MAGNUS® (purchased by Sidus S.A. Buenos Aires, Argentina) containing 25 mg sildenafil were weighed and finely powdered. Portions of the powder equivalent to 25 mg of sildenafil were dissolved in water and solid residues were separated by filtration. Solutions were then transferred to 100 mL flasks, and diluted to the final volume with ultra pure water. Solutions were diluted 1:100 and conditioned to pH 11 with NaOH(d), leading to a final sildenafil concentration of 2.5 μg mL^{-1}.

2.3. General procedure and FIA manifold

A pre-concentration column (a glass column adapted from a Pasteur pipette with 0.6 cm diameter and 5 cm length) was filled with Amberlite XAD-1180, previously activated. After being thoroughly washed with bi-distilled water, the column was connected to the FIA manifold (Fig. 1).

In order to coat the resin with surfactant, valve 1 was fixed in position 1, allowing the stream of HTAB solution (1×10^{-3} mol L^{-1}) to flow through the column for about 30 s, while Standard/Sample solution (previously pre-conditioned to pH 11) filled the sample coil (valve 2, position 1). For sildenafil pre-concentration, valve 2 was changed to position 2, allowing the stream of Standard/Sample solution to flow into the column. After that, the stream of HTAB was replaced by NaOH (pH 11) to remove the sample matrix (for 1 min). At this stage, the replacement of NaOH stream could be made either manually or using an additional 3-port valve. Finally, Sildenafil elution was achieved switching valve 2 to position 1 and valve 1 to position 2, allowing the stream of ethanol through the column (30 s). Fluorescence emission of the eluate containing sildenafil–HTAB was recorded by a spectrofluorimeter at λ_{exc}=310 nm and λ_{em}=430 nm.

After elution, valve 1 was switched to position 1 (initial position) in order to regenerate the column for the next determination. The time of analysis was 2 min, including the column regeneration step.

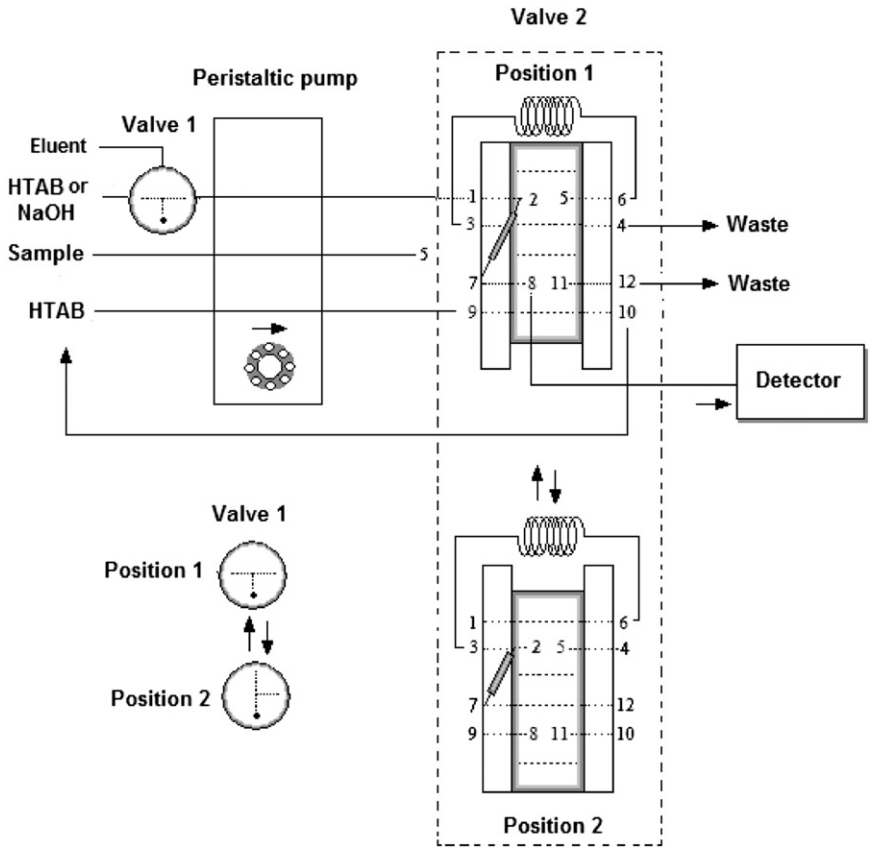

Fig. 1. FI manifold for the solid-phase extraction and fluorescence analysis of sildenafil.

3. Results and discussions

The polymeric XAD resins are used for adsorption of a wide variety of substances from aqueous systems and polar solvents. Depending on the chemical composition, porosity and polarity, there are many XAD resins commercially available. Some of the most commonly used resins are XAD-2, XAD-4, XAD-7, XAD-16, XAD-200, XAD-1180 and XAD-2010.

The retention capacity for hydrophobic compounds depends on its nature and molecular weight (MW). Therefore, increasing the MW of hydrophobic compound the order of adsorbents XAD-2 < XAD-4 < XAD-16 < XAD-1180 is recommended. On the other hand, XAD-7 is the only moderately polar resin available to adsorb moderately polar to polar compounds, being widely used in the pharmaceutical industry [34].

The adsorption process takes place on the XAD resin bead consisting of an agglomeration of many small microspheres, resulting in a continuous gel phase and continuous pore phase. The open-cell porous structure allows water to penetrate the pores easily. In the adsorption process, the hydrophobic portion of the adsorbate molecule is preferentially adsorbed on the hydrophobic surface of the resin, while the hydrophilic section of the adsorbate remains oriented to the aqueous phase.

In our attempts to adsorb sildenafil on XAD resins, XAD-2, XAD-4, XAD-7, XAD-16 and XAD-1180 resins were tested. All tested resins showed null retention for sildenafil, except XAD-7 which showed poor retention.

In order to improve the retention capacity, resins were modified with SDS anionic surfactant and HTAB cationic surfactant. The adsorbed surfactant layer on the resins would modify the interaction profiles of sildenafil since the analyte instead of being partitioned between aqueous and resin solid phase, would be partitioned between the aqueous phase and adsorbed surfactant layer. Hence, the partition coefficient of sildenafil was improved by the adsorbed surfactant layer. Within tested XAD resins (mentioned above), the modified surfactants–XAD-1180 resin was the most effective. The rest of tested resins showed insignificance improvement in their retention capacity in the presence of HTAB.

Above pH 10.5, the sildenafil molecule adopted negative charge due to the NH–amide group dissociation [35]. The negatively charged molecule interacted with agglomerates of HTAB by electrostatic force. On the other hand, below pH 7 sildenafil molecule was positively charged and thus, was attracted by SDS molecules. However, modified HTAB–(XAD-1180) resin had the additional advantage of fluorescence enhancement due to the micelar environment (Fig. 2).

Although previous studies showed that the interaction between monomers of SDS and sildenafil form a complex sildenafil–$(SDS)_n$, which produces fluorescence enhancement [26] in the presence of ethanol this complex is disrupted. Therefore, sildenafil could be separated and pre-concentrated by modified SDS–(XAD-1180) resin, but no additional fluorescence enhancement was observed as occurs in the HTAB–sildenafil system.

Experimental studies concluded that HTAB–(XAD-1180) resin was the most adequate system for sildenafil pre-concentration. The presence of ethanol as eluent produces a rapid elution of sildenafil–HTAB though it modified critical micelar concentration (CMC) of HTAB [36]. Consequently, the use of ethanol slightly attenuated the fluorescence signal of sildenafil.

3.1. Influence of pH

Though sildenafil has acid–base properties, its native fluorescence in aqueous solution is scarcely affected by varying pH values. But it is expected that the pH has influence on the

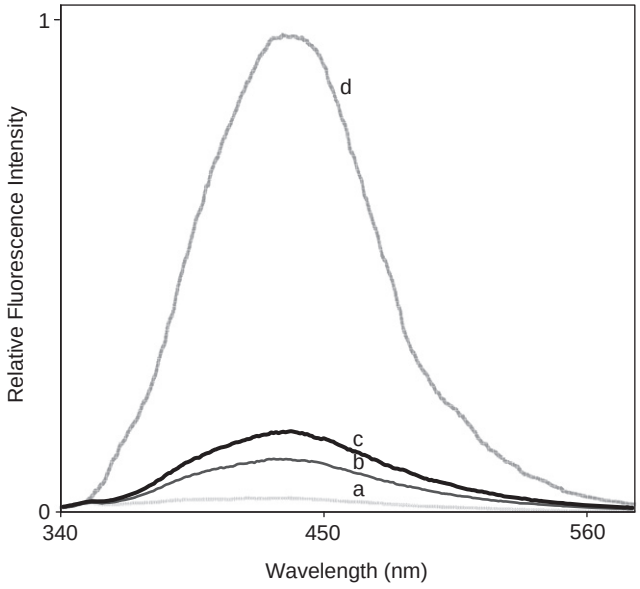

Fig. 2. Fluorescence emission spectra of sildenafil–HTAB. C_{HTAB} for $a=0.0$ mM, $b=0.5$ mM and $c=1.0$ mM; $C_{sildenafil}=40\ \mu g\ mL^{-1}$; pH 11. "*d*" is "*c*" signal after pre-concentration. $\lambda_{exc}=310$ nm; $\lambda_{em}=435$ nm.

Table 1
Optimization of FIA variables.

Variables	Studied range	Optimum value
Column length (cm)	0.30–10.00	5.00
Column diameter (cm)	0.60–1.00	0.60
HTAB flow rate (mL min^{-1})	1.00–4.00	2.00
NaOH at pH 11 (mL min^{-1})	1.00–4.00	2.00
Eluent flow rate (mL min^{-1})	1.00–4.00	1.00
Sample coil volume (mL)	0.50–10.00	1.00–3.00
XAD-resin dry weight (g)	–	0.35

interaction equilibrium between HTAB and the analyte, in which mainly electrostatic force of attraction is involved. At alkaline pH molecule of sildenafil adopts negative charge; this fact favors its interaction with cationic surfactant such as HTAB [25]. Therefore, increasing pH improves the retention capacity of HTAB coated resin for sildenafil and consequently, greater fluorescence signal for the final eluate was observed. Hence, the working pH value for the sildenafil solution/sample is fixed at 11.

3.2. Optimization of FIA variables

In order to obtain the best sensitivity and reproducibility, variables which influence performance of the on-line method were studied and optimized (Table 1). The most important variables were the length and diameter of the pre-concentration column, the amount (mg) of XAD-1180 resin used to fill the column and the reagents flow rates.

When column length increased, it improved its retention capacity; increase of column diameter produced a tailed signal due to the diffusion process.

The amount of resins influences the compaction of the column. Non-compacted column creates air bubbles and solvent lakes which produce tailed residual signals on fiagram. To minimize diffusion processes, resin must be well compacted.

Finally, the reagents flow rate affects the interaction time between the analyte and adsorbent to reach equilibrium. However, low flow rates lead to longer sampling time. So, a flow rate of 1 mL min^{-1} was chosen as optimal.

The eluent selection was realized taking into account the best fiagram profile obtained. Different solvents were tested in order to achieve a rapid, effective and complete elution of sildenafil from the resin. The use of long-chain organic solvents was avoided considering the potential risk for environment and the operator. Polar solvents such as ethanol and methanol were effective for this purpose; ethanol has been finally chosen due to its lower toxicity and desorption effectiveness.

3.3. Figures of merit

FI calibration curves of sildenafil (Fig. 3) were performed under optimal conditions according to general procedure. Data were fitted by standard least-squares treatment; all analytical parameters are given in Table 2. Detection limit (LOD) and quantification limit (LOQ) were obtained with excitation and emission slit widths of 5.0 and 10.0 nm, respectively. LOD was estimated as the concentration of analyte which produced an analytical signal equal to 3 times the standard deviation (3 SD) of the blank and LOQ=10 SD. In order to compare the present method with others reported, Table 3 shows LODs values for sildenafil determination.

3.4. Validation and applications

In order to study the accuracy, recovery studies were carried out by the standard addition method. Known amounts of analyte were added to urine samples and herbal medicine infusion for ED

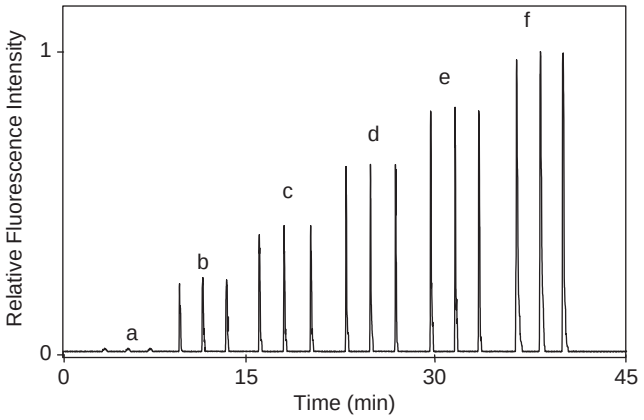

Fig. 3. Calibration curve of sildenafil. Standard solution of sildenafil: (a) 0.00 μg mL^{-1};(b) 1.00 μg mL^{-1}; (c) 2.00 μg mL^{-1}; (d) 3.00 μg mL^{-1}; (e) 4.00 μg mL^{-1}; and (f) 5.00 μg mL^{-1}. $\lambda_{exc}=310$ nm; $\lambda_{em}=430$ nm. Excitation and emission slit widths are 3 nm and 5 nm, respectively.

Table 2
Analytical parameters of spectrofluorimetric developed methods and UV–visible spectrophotometric determination of sildenafil.

Analytical parameters	UV–visible photometry	HTAB-mediated fluorimetry	This method
λ_{max} (nm)	225	$\lambda_{exc}=290$ $\lambda_{em}=435$	$\lambda_{exc}=310$ $\lambda_{em}=430$
LOL (μg mL^{-1})	5.6–50.0	(0.004–25)[a,b]	(0.0005–10.5)[a,b]
Slope	0.0441	67.50[a]	495.10[a]
Intercept	0.03	45.47[a]	24.33[a]
Correlation coefficient	0.997	0.998[a]	0.980[a]
SD of blank ($n=6$)	0.026	0.029	0.033
LOQ (μg mL^{-1})	5.60	0.004	0.00066
LOD (μg mL^{-1})	1.76	0.0012	0.0002

[a] Excitation slit width=5 nm; emission slit width=10 nm.
[b] Excitation slit width=3 nm; emission slit width=5 nm.

Table 3
Analytical parameters of reported and proposed methods for sildenafil determination.

Method	Detection system	LOL ($\mu g\ mL^{-1}$)	LOD ($\mu g\ mL^{-1}$)	References
Extractive spectrophotometric methods	UV–visible spectrophotometry	Method A 1.25–25 Method B 1.5–60	0.16 0.18	[5]
HPLC	UV–visible spectrophotometry	0.01–1	Not available	[8]
HPLC–MS	Electrospray positive ionization (ESI) mass-spectrometry	0.000125–0.04	0.00005	[11]
Micelar electrokinetic chromatography	UV–visible spectrophotometry	0.080–0.9	0.017	[12]
Adsorptive stripping square–wave voltammetry	Voltammetry	0.029–0.32	Not available	[13]
Polymer membrane sensors	Potentiometry	6.6–600	3.3	[14]
Surfactant-mediated spectrofluorimetry	Spectrofluorimetry	Method A 0.004–25 Method B 0.005–50	0.0002 0.0016	[18]
This method	FI–fluorimetry	0.0006–10.5	0.0012	–

Table 4
Recovery study of sildenafil in urine and mixture herbal extract.

Samples	Added ($\mu g\ mL^{-1}$)	Found ($\mu g\ mL^{-1}$) ± RSD (%)[a]	Recovery (%)[a,b]
Solution of tablets ($2.5\ \mu g\ mL^{-1}$)	0.250	2.761 ± 1.9	100.4
	0.500	2.897 ± 2.0	96.5
	0.750	3.260 ± 1.6	100.3
	1.000	3.478 ± 1.4	99.4
	1.250	3.755 ± 1.3	100.1
	1.500	4.140 ± 1.1	103.5
Mixture herbal extract	0.250	0.259 ± 2.2	103.6
	0.500	0.505 ± 2.0	101.0
	0.750	0.746 ± 1.9	99.4
	1.000	0.998 ± 1.4	99.8
	1.250	1.267 ± 1.1	101.3
	1.500	1.489 ± 1.6	99.2
Urine	0.250	0.257 ± 2.1	102.8
	0.500	0.486 ± 1.9	97.2
	0.750	0.758 ± 2.0	101.0
	1.000	1.015 ± 1.1	101.5
	1.250	1.240 ± 1.1	99.2
	1.500	1.511 ± 1.7	100.7

[a] Average of 3 replicates.
[b] $100 \times [(\text{found} - \text{base})/\text{added})]$.

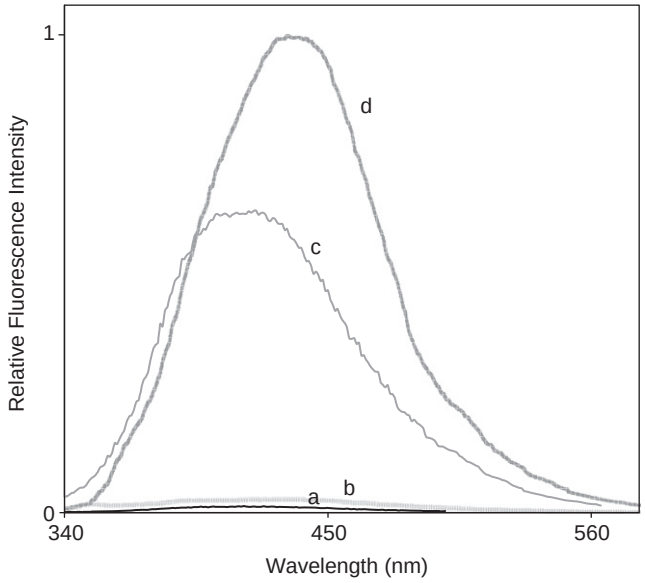

Fig. 4. Application of the methodology to determine sildenafil in urine. (a) Urine sample treated by surfactant coated XAD-1180 resin; (b) Aqueous solution of sildenafil ($40\ \mu g\ mL^{-1}$); (c) Fluorescence emission of urine (dilution 1:10) and (d) Urine spiked with sildenafil ($40\ \mu g\ mL^{-1}$) treated as described in the general procedure.

treatment. Results showed quantitative recoveries (Table 4), indicating good accuracy of the proposed procedure.

The results obtained from the herbal medicine infusion were compared to the HPLC official method [15,35,37]. The obtained regression close to 1 ($y=0.976x+0.079$, for $n=10$) indicates a satisfactory correspondence between the two methods. In addition, the coefficient of determination (R^2 is 0.9999) indicates a strong correlation between the methods. Another indication of the very good relation between these two methods is the fact that the confidence intervals for slope and intercept include 1 and 0, respectively. The residual plot shows a random arrangement which has been attributed to an accuracy of fit of the model and homoscedasticity in the linear range of 1–10 $\mu g\ mL^{-1}$.

Besides we have studied the correspondence between the methods by the Elliptical Joint Confidence Region (EJCR) and the confidence region for slope and intercept showed that the values 1 and 0 for the slope and the intercept are found inside the elliptical region.

Take into account that urine's matrix is highly fluorescent; in Fig. 4 spectra of urine with and without pre-concentration step are presented. The obtained results suggest that the developed method for eliminating interference was efficient. The advantage of the described procedure for analysis of sildenafil in urine is the simplicity of the sample pretreatment, allowing a high sampling rate (30 samples per hour). Hence, the developed methodology could be very useful in routine clinical analysis.

Due to the selective retention capacity of the surfactant coated resin, at working conditions most of the concomitant polar compounds of studied sample's matrixes were not adsorbed and were removed during the washing step. When dyes, pigments and non-polar compounds were adsorbed on resin in the presence of sildenafil, they have much longer elution time, in order that the simultaneous elution (interference) did not occur. This could be attributed to the differential partition coefficient for each specie.

4. Conclusions

On-line pre-concentration of sildenafil with FI-fluorimetric detection was proposed in the present work. Different adsorbent XAD polymeric resins were studied in order to evaluate the retention capacity for sildenafil. HTAB coated XAD-1180 resin proved effective for sildenafil retention in alkaline medium and therefore, it was used for on-line separation and pre-concentration. A quick and effective elution of sildenafil–HTAB was achieved employing ethanol as the eluting agent. The eluate was then flowed to the spectrofluorimeter and the fluorescence was measured. The micelar environment provided by the surfactant gave an additional advantage to the sensitivity of the method, enhancing the fluorescence emission of sildenafil.

Compared to the native fluorescence of sildenafil, the proposed method presented a remarkable improvement in sensitivity (33-fold increase on its fluorescence emission), permitting sildenafil determination at 0.2 ng mL^{-1}. This method is simple, sensitive, accurate and precise allowing the analysis of sildenafil in tablets, urine and herbal medicine. Sample pretreatment before analysis was neither involved nor necessary for the proposed method, increasing sampling time up to 30 h^{-1}.

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