ORIGINAL ARTICLE

Determination of sildenafil by preconcentration on surfactant coated polymeric resin followed by spectrofluorimetry ☆

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Abstract The illicit addition of phosphodiesterase type-5 (PDE-5) inhibitors like sildenafil (Viagra) in product offered as herbal medicine or dietary supplement for male erectile dysfunction has concerned authorities in recent times. In this paper, we proposed a sensitive surfactant-coated Amberlite XAD™ resin for sildenafil preconcentration method with spectrofluorimetric detection. Retention capacity of micellar coated XAD resin for sildenafil was studied and the obtained eluate was measured by spectrofluorometer at excitation and emission wavelengths of 350 and 430 nm, respectively. This method allowed the detection of sildenafil at 0.15 ng/mL with linear range of 0.0003–7.0 μg/mL. The method has been successfully applied to the analysis of some local commercially available herbal medicines and urine.

1. Introduction

Natural or herbal medicines treatments for erectile dysfunction (ED) are a popular alternative or adjunct to treatment with traditional pharmacologic agents. While the debate regarding the potential benefits and risks of herbal medications is ongoing, and their efficacy continues to be examined, a significant number of these products are adulterated with undeclared synthetic drugs. Clinicians and patients alike should be aware of the possibility that herbal medicines may contain active ingredients not listed on packaging, which may result in pharmacologic interactions and unanticipated side effects.

It is commonly believed that natural ingredients are inherently safer and healthier than synthetic ingredients [1]. Products sold as "dietary supplements" are subject to significantly less regulation and are often not required to have safety testing or regulatory bodies' approval before they enter the market. Without adequate safeguards and quality control mechanisms, there is no assurance that products are free of undeclared medications or contaminants. In fact, many investigators have shown that other purportedly "all-natural" dietary supplements claimed to improve sexual function actually contain synthetic PDE-5 inhibitors like sildenafil or its analogs [2]. Therefore, analytical chemistry plays an important role in the quality control in the pharmaceutical industry for quantifying not only the claimed contents but also impurities and/or adulterants that may be presented in the medicaments [3].

Most herbal medicines and their derivative products are often prepared from crude plant extracts, which comprise a complex mixture of different phytochemical constituents (plant secondary metabolites). The chemical features of these constituents differ considerably among different species. Therefore, the qualitative analysis of herbal medicines is described as "complex system research", which is really a challenging task for scientists [4].

Fluorescence-based methodologies for quality control in pharmaceutical and clinical monitoring have the advantages of being highly sensitive and selective, besides cheap in equipment [5–7].

Sildenafil is a selective inhibitor of cyclic guanisine monophosphate specific PDE-5. It is the first effective synthetic oral therapy for treatment of erectile dysfunction with potent vasodilatative effect. Since its approval by the FDA in March 1998, a large number of prescriptions for this drug, known as Viagra, have been filled; 148 million pills have been used in Europe and more than one billion worldwide to fill about 130 million prescriptions [8–9]. The worldwide sales of sildenafil have been steadily increasing since then, reaching an economic impact of about 5 billion dollars just for 2011 [10].

Severe cardiovascular events—some leading to death, such as myocardial infarction, ventricular arrhythmia, cardiac arrest, transient ischemic attack, and hypotension—have been reported post-marketing in temporal association with the use of sildenafil. In most cases, pre-existing cardiovascular risk factors were noted [11–12]. The particular social importance of the problem of erectile dysfunction, the effectiveness of the drug and the ease of access to sildenafil (even without medical prescription) have led to cases of uncontrolled use [13–16]. This is especially dangerous for patients with pre-existing cardiovascular risk and/or taking concomitant medicines for cardiovascular diseases.

The needs for an adequate determination method to detect the presence of sildenafil in widespread formulations have lead to the development of several analytical methodologies [17–23] (Table 1). In our recent work, we have reported for the first time a fluorescence method for sildenafil determination [23]. The fluorescence-based methodology for sildenafil detection has the advantage over the traditional detecting method (UV–visible) in sensitivity, inherent to the technique itself. Moreover, the use of surfactants has greatly enhanced the fluorescence emission of sildenafil as well as enlarged the linear range of concentration [23].

Table 1 Comparison of reported methods for sildenafil determination.

Method	Detection system	Linearity range (μg/mL)	LOD (μg/mL)	Reference
Extractive spectrophotometric method	UV–visible spectrophotometry	Method A 1.25–25 Method B 1.5–60	0.16 0.18	[16]
HPLC	UV–visible spectrophotometry	0.01–1	No available	[17]
Micellar electrokinetic chromatography	UV–visible spectrophotometry	0.080–0.9	0.017	[18]
HPLC-MS	Electrospray positive ionization (ESI) mass-spectrometry	0.000125–0.04	0.00005	[19]
Adsorptive stripping square-wave voltammetry	Voltammetry	0.029–0.32	No available	[20]
Polymer membrane sensors	Potentiometry	6.6–600	3.3	[21]
Surfactant-mediated spectrofluorimetry	Spectrofluorimetry	Method A 0.004–25 Method B 0.005–50	0.0012 0.0016	[22]
Present method	Spectrofluorimetry	0.0003–7	0.00015	–
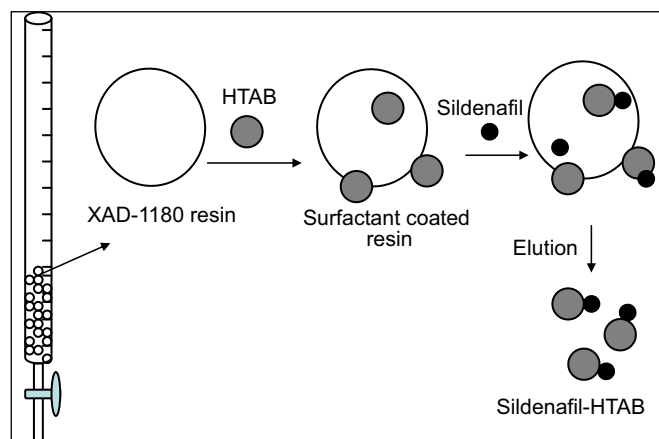
Fig. 1 Schematic representation of the preconcentration step in XAD column.

In the present paper, hexadecyltrimethylammonium bromide (HTAB) coated XAD resin has been introduced for sildenafil pre-concentration in batch system in order to increase the sensitivity and selectivity of the method for determining sildenafil in herbal medicines infusion and urine at trace level.

2. Experimental

2.1. Instrumentals

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

A Beckman coulter UV/VIS spectrophotometer model DU640 with 10 mm optical path cells was used to record UV–vis absorption spectra.

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

2.2. Reagents

Sildenafil (as citrate) was kindly provided by Gador S.A. (Buenos Aires, Argentina). Reagents of analytical grade were used: HTAB was purchased from Tokyo Kasei Industries (Chuo-Ku, Tokyo, Japan); Tris (Mallinckrodt Chemical Works, New York, Los Angeles, St. Louis, USA); NaOH and HCl (Merck, Darmstadt, Germany). High-purity water was obtained from a Millipore (Milford, MA, USA) Milli-Q Plus System.

2.3. Assay solutions

Sildenafil standard solution containing 2.0 mg/mL was prepared by weighing 50 mg of sildenafil citrate and dissolving it in a 50 mL flask with ultra-pure water. This solution was stable for at least two weeks and stored at room temperature. 5.0 mM HTAB was prepared by dissolving the reagent in ultra-pure water. The eluent was prepared from a mixture of ethanol/phosphoric acid solution (200 mM) (1:1).The pH values were adjusted by the addition of solutions of NaOH(c) or HCl(conc.) until the target pH value was reached.

2.4. Sample solutions

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

An aqueous extract of herbal mixture (*Haploppapus baylahuen*, *Lycopodium 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 boiling ultra-pure water. After 5 min, the mixture was filtered and made to 100 mL with ultra-pure water.

2.4.2. Human urine
Fresh matinal human urine was obtained from healthy volunteers. The urine samples were centrifuged and the supernatants were collected in sterile containers and stored at $-5°$ C until assaying.

2.5. General procedure

Amberlite XAD resin (2 g) previously activated by washing step with ethanol/HCl (60:40) was placed into a glass column (burette of 25 mL of capacity). The resin was modified with surfactant by passing 10 mL (0.1 M) of HTAB; the residue was removed by washing the column with double distilled water. For sildenafil determination, standard/sample solution (preconditioned to pH 11) was flowed through the column and then washed with aqueous solution of NaOH at pH 11 in order to remove the matrix. The adsorbed analyte was then eluted with 5 mL of ethanol/phosphoric acid (Fig. 1) and the middle portion of 3 mL was collected and analyzed by spectrofluorimetry (λ_{ex} 310, λ_{em} 430 nm).

3. Results and discussions

Micelles are highly dynamic aggregates and rates of uptake of monomers into micellar aggregates are nearly within the
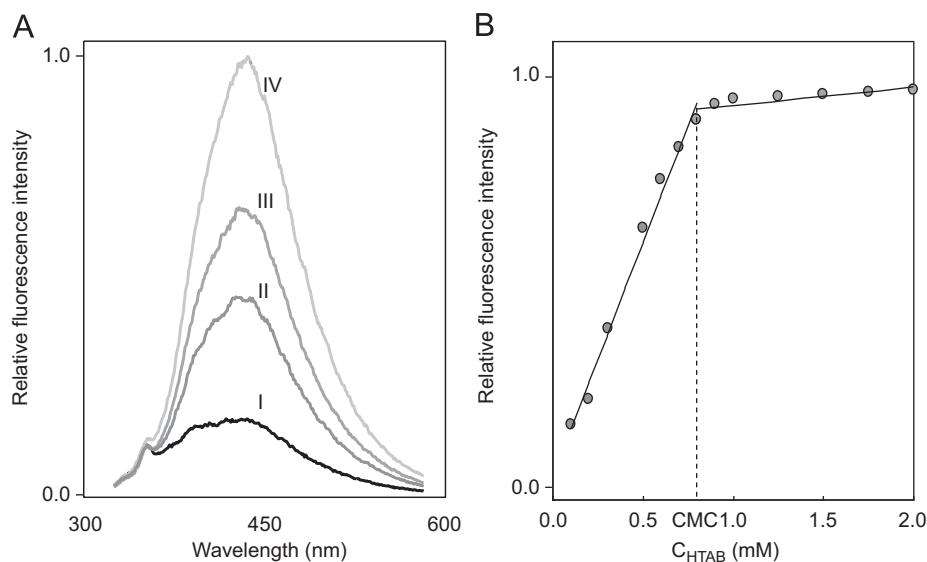
Fig. 2 (A) Fluorescence emission of sildenafil–HTAB. (B) Determination of CMC for HTAB–sildenafil system. (A) Spectra of sildenafil in the absence and presence of HTAB; $C_{sildenafil}=40$ μg/mL; C_{HTAB} (mM): I=0.00, II=0.40, III=0.60, IV=1.00. (B) Sildenafil fluorescence at increasing concentration of HTAB; $C_{sildenafil}=40$ μg/mL;

diffusion-controlled limit [24]. When surfactant concentration in water is very low, these monomers behave like independent molecular entities. As the concentration is increased, they tend to come close to each other and form aggregates of different sizes and shapes. The formed micellar microenvironment is still an object of study due to its peculiar behavior and capable of modifying a series of physical–chemical properties of solutes in the solution like solubility and catalysis activity, among others [25]. For some species, the micelle core can provide an enhancement of their fluorescent emission due to the viscosity of the micellar microenvironment, thus restricting the free rotation of ligands of the molecule which competes for emission pathway [26–27]. Sildenafil has a basic functional group (NCH_3--piperazine) with a pKa value of 8.7 and a second pKa of 9.6–10.1 due to NH-amide. Based on its acid–basic characteristics, sildenafil can adopt positive or negative charge, depending on the pH of the medium. At alkaline pH, sildenafil has negative charge which in micellar solution of cationic surfactant of HTAB interacts with positively charged micelles.

3.1. Spectral behavior of sildenafil–HTAB system

The fluorescence spectra of sildenafil in the absence or presence of different concentrations of HTAB at pH 11 are shown in Fig. 2. a. When cationic surfactant HTAB is added above a certain limit, the positively charged micellar aggregates begin to form. The micellar interface with positive zeta-potential would be the preferred location for negatively charged molecule of sildenafil to bound, through the electrostatic force of attraction [28]. With further addition of HTAB, the aggregates grow until reaching the CMC value, where micelles coexist with surfactant monomers [29]. When the monomer concentration is far from CMC value, the fluorescent intensity does not increase significantly with varying C_{HTAB}. But near the CMC the fluorescence signal of sildenafil is enhanced, indicating that drug molecules are bound to the micellar aggregates. The corresponding

fluorescence enhancement reaches its maximum when HTAB micelles are formed ($C_{HTAB}=$CMC) (Fig. 2b).

3.2. Surfactant coated XAD resin

XAD resins are a series of macro-reticular polymeric resins with varying physical properties (e.g. polarity, pore size, and density) depending on their chemical nature. Most of the commercially available resins are non-polar, widely used in chemical, pharmaceutical and food industries to adsorb non-polar substances for purification step. These resins have the advantages for being highly resistant to extreme working conditions (e.g. pH and pressure) and economical as they are recyclable.

In our first attempt to adsorb sildenafil directly onto the resins (XAD-2, -4, -7, -16, -1180) and showed limited or no retention. This could be attributed to the fact that apolar nature of the most studied resins is not suitable for adsorbing a polar compound as sildenafil. Nevertheless, the use of HATB coated XAD resin (especially for XAD-1180) showed a good retention of sildenafil from aqueous medium. Moreover, the obtained eluate presented an additional fluorescence enhancement, due to the micellar microenvironment provided by aggregates of HTAB which eluated simultaneously with sildenafil.

3.3. Influence of pH and surfactant concentration

Although sildenafil has acid–basic properties, the native fluorescence emission is insignificantly affected by the change in pH. However, it is expected that the pH parameter can influence its interaction with the cationic surfactants, and therefore, modify the retention capacity of the surfactant coated resin. Fig. 3 shows the influence of pH on sildenafil retention onto HTAB-coated XAD-1180 resin, which at alkaline values increases the retention of sildenafil. In Fig. 4 the emission spectra of sildenafil before and after

preconcentration are shown. The preconcentration factor plus the emission enhancement due to the presence of HTAB have lead to a great fluorescence enhancement.
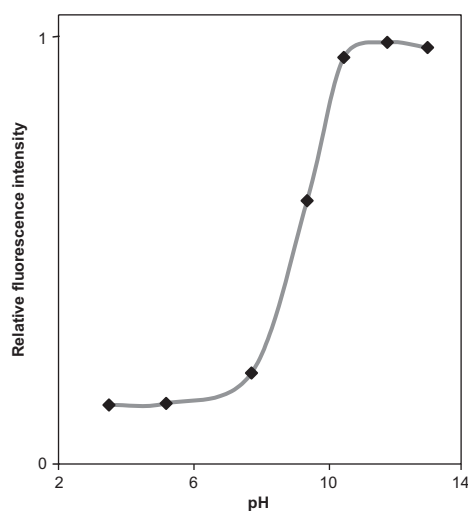
Fig. 3 Influence of pH on sildenafil–HTAB interaction $C_{\text{Sildenafil}} = 5\ \mu g/mL$.
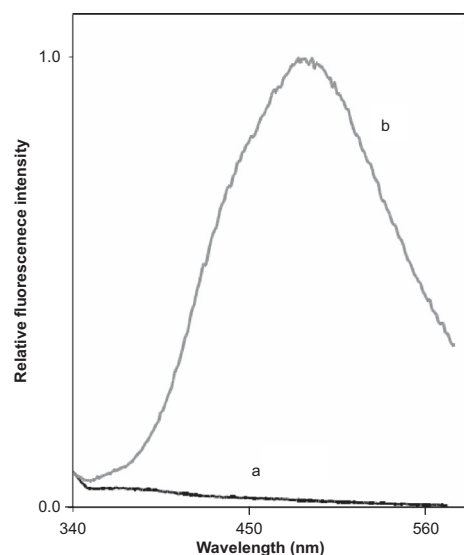
Fig. 4 Emission spectra of sildenafil before and after preconcentration. $C_{\text{sildenafil}} = 5\ \mu g/mL$ where: (a) before preconcentration step and (b) after preconcentration step (initial volume $= 200$ mL).

3.4. Figures of merit

Calibration curves for sildenafil were prepared under optimal conditions according to the general procedure as described under Experimental section. Data were fitted by standard least-squares treatment and the analytical parameters determined for each method are given in Table 2. According to IUPAC definition, the slope of calibration graph (m) represents the sensitivity of calibration. Compared with spectrophotometric detection for sildenafil assay, the proposed method achieved enhancement on sensitivity of approximately 1000 fold. These facts show the potential of the proposed method to compete with the UV–vis spectrophotometry for sildenafil assay, which is the official method for this purpose.

3.5. Validation and application of methods

In order to study the accuracy, recovery studies were carried out by standard addition method. Known amounts of sildenafil at different concentration levels were added to herbal medicines infusions and urine, which were then determined. Results are shown in Table 3. Average percent recoveries obtained were quantitative, indicating good accuracy of the proposed procedures. The spectrofluorimetric detection system has been already validated against the official method and yielded satisfactory results [22]. The highly complex matrices did not interfere with the measurement of the analyte.

4. Conclusions

In this paper, sildenafil has been preconcentrated by HTAB coated resin and the eluate has been determined by spectrofluorimetry. Since sildenafil could adopt cationic or anionic form, the working pH is a fundamental parameter for its interaction with surfactants, and thus affects the preconcentration factor. Moreover, the use of surfactants provides a simple means to enhance the fluorescence of sildenafil, increasing the sensitivity of the method. The matrix of studied samples did not interfere the determination for sildenafil, demonstrating the effectiveness of the present methodology for removing potential interference from the matrix by washing steps. The proposed method requires simple reagents and low-cost instruments. Additionally, the column can be regenerated easily in order to perform a great number of analyses, which is ideal for large-scale routine quality control.

Table 2 Analytical parameters and figures of merit of different optical methodologies for sildenafil determination.

Analytical parameters	UV–vis photometry	HTAB-mediated fluorimetry [22]	This methodology
$\lambda_{\max}$ (nm)	225	$\lambda_{\text{exc}} = 290$ $\lambda_{\text{em}} = 435$	$\lambda_{\text{exc}} = 310$ $\lambda_{\text{em}} = 430$
Linearity range (µg/mL)	5.6–50.0	(0.004–25)	(0.0003–7)
Slope	0.0441	67.50	655.20
Intercept	0.03	45.47	30.11
Correlation coefficient	0.997	0.998	0.990
LOQ (µg/mL)	5.60	0.004	0.0003
LOD (µg/mL)	1.76	0.0012	0.00015

Table 3 Recovery study for sildenafil in real samples of herbal medicines.

Samples	Added (µg/mL)	Found (µg/mL)	RSD (%)	Recovery (%)[a]
Lycopodium saururus extract	0.250	0.248	±2.7	99.2
	0.500	0.505	±2.5	101.0
	0.750	0.754	±2.1	100.5
	1.000	1.010	±1.4	96.2 99.6
	1.250	1.245	±1.6	
	1.500	1.502	±0.9	100.1
Extract of mixture herbals	0.250	0.251	±3.1	100.4
	0.500	0.501	±1.9	100.2
	0.750	0.749	±2.0	99.8
	1.000	1.009	±1.1	100.9
	1.250	1.241	±1.9	99.3
	1.500	1.509	±2.0	100.6
Urine	0.250	0.260	±2.8	104.0
	0.500	0.521	±1.2	104.2
	0.750	0.756	±1.1	100.8
	1.000	1.012	±1.0	101.2
	1.250	1.251	±1.3	100.0
	1.500	1.514	±2.1	100.9

[a]Average of 6 replicates.

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