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Determination of erythromycin in urine and plasma using microbore liquid chromatography with tris(2,2′-bipyridyl)ruthenium(II) electrogenerated chemiluminescence detection

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Abstract

Erythromycin is determined in both urine and plasma samples using microbore reversed-phase liquid chromatography with tris(2,2′-bipyridyl)ruthenium(II) [$Ru(bpy)_3^{2+}$] electrogenerated chemiluminescence (ECL) detection. $Ru(bpy)_3^{2+}$ is included in the mobile phase thus eliminating band broadening caused by post-column reagent addition. Extra column band broadening is an important concern in microbore liquid chromatography due to the small peak volumes. Erythromycin was studied in both water and biological samples. The detection limit for erythromycin in standards is 0.01 μM or 50 fmol injected with a S/N of 3 and a linear working range that extends four orders of magnitude. Human urine and blood plasma were also studied. Urine samples were diluted and filtered before injection. Ultrafiltration was used to remove protein from blood plasma samples prior to injection. Erythromycin was selectively detected in the body fluid samples without any further sample preparation. The detection limits obtained for erythromycin in urine and plasma are 0.05 and 0.1 μM, respectively, for 5 μl injected on a 150×1 mm I.D. C_{18} column. © 1997 Elsevier Science B.V.

Keywords: Erythromycin; Tris(2,2′-bipyridyl)ruthenium(II)

1. Introduction

Erythromycin is an important macrolide antibiotic that is used for treatment of bacterial infections in humans and animals. At this time, there still exists a need for improved detection techniques of erythromycin and related macrocyclic antibiotics. In most cases, erythromycin has been detected electrochemically [1–6], but it can also be detected with UV absorbance [7] and with fluorescence [8–11]. At wavelengths above 220 nm, erythromycin is transparent except for a weak chromophore at 280 nm, and although the molar absorptivity is considerably higher at wavelengths below 220 nm, other problems occur due to interferences of species co-extracted from biological samples [8]. Stubbs et al. reported limits of detection of 0.25 and 1 μg/ml for erythromycin in serum and urine, respectively, using UV absorbance detection at 200 nm. In order to use fluorescence detection, post-column derivatization is necessary. Khan et al. obtained detection limits for erythromycin of 12.5 ng/ml in plasma and 50 ng/ml

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in urine using a post-column ion-pair extraction with the strongly fluorescent 9,10-dimethoxy-anthracene-2 sulfonate [8]. Limits of detection using electrochemical detection have been reported as low as 5–10 ng/ml in plasma [2]. One drawback associated with all of these techniques is the sample preparation required when assaying erythromycin in biological samples. Due to interferences from other compounds present in complex biological samples, it is necessary to do an extraction and in some cases a derivatization prior to the determination of erythromycin.

In recent years, chemiluminescence (CL) has become a very attractive detection system for liquid chromatography due to the very low detection limits, wide linear dynamic range and relatively simple instrumentation [11,12]. One commonly used CL system is tris(2,2′-bipyridyl)ruthenium(III) [$Ru(bpy)_3^{3+}$] CL. $Ru(bpy)_3^{3+}$ CL has proven to be a very sensitive detection system for compounds which contain a secondary or tertiary amine. Compounds which $Ru(bpy)_3^{3+}$ CL has been applied to include amino acids [13–16], antihistamines [17], clindamycin antibiotics [18], glyphosate [19], NADH [20,21], dansyl derivatized amino acids [22,23] and oxalate [22,24]. $Ru(bpy)_3^{2+}$ CL has also previously been applied to the detection of erythromycin, which contains a tertiary amine, giving a detection limit of 24 ng/ml [25].

$Ru(bpy)_3^{2+}$ CL is based on the oxidation–reduction reaction mechanism postulated by Rubinstein et al. [26].

$$Ru(bpy)_3^{2+} \rightarrow Ru(bpy)_3^{3+} + e^-$$

$$Ru(bpy)_3^{3+} + \text{reductant} \rightarrow \text{product} + [Ru(bpy)_3^{2+}]^*$$

$$[Ru(bpy)_3^{2+}]^* \rightarrow Ru(bpy)_3^{2+} + h\nu$$

The $Ru(bpy)_3^{3+}$ can either be generated externally by bulk electrolysis and mixed with the analyte, or it can be generated in situ at an electrode surface. This second method is termed electrogenerated chemiluminescence (ECL) [27]. The CL intensity obtained is directly proportional to the amount of reductant or analyte present.

$Ru(bpy)_3^{3+}$ CL can be used for detection in LC using one of three different methods. The first two methods involve adding $Ru(bpy)_3^{2+/3+}$ post-column. $Ru(bpy)_3^{3+}$ can be generated externally and then mixed with the eluent from the column before entering the detection cell where the CL emission is measured [16–18,28], or $Ru(bpy)_3^{2+}$ can be mixed with the eluent from the column before entering the detection cell where it is then oxidized at an electrode surface to $Ru(bpy)_3^{3+}$ and reacts with the analyte to give light emission [13,23,24,29]. Both of these methods have the disadvantages associated with post-column reagent addition which include band broadening, sample dilution and the need for additional instrumentation. The final method involves incorporating $Ru(bpy)_3^{2+}$ in the mobile phase [13,19,22]. This method offers the advantage over the post-column addition methods of better detection limits and wider linear dynamic range [22]. It also makes it more feasible for use as a detection system in microbore LC.

Microbore liquid chromatography has proven to be a useful and important separation technique that has been growing in use over the past several years, as is evident by the many recent publications using this technique [30–35]. This technique offers many important advantages over conventional liquid chromatography which include increased separation efficiencies, improved mass sensitivity, the ability to work with smaller sample sizes and conservation of exotic and expensive mobile phases due to the use of low flow-rates [36]. Because of the small sample sizes and low flow-rates used with microbore LC, the limiting of band broadening is critical. At this time there has been only one publication applying $Ru(bpy)_3^{2+}$ CL for detection in microbore LC [37]. In that paper, $Ru(bpy)_3^{3+}$ was added post-column which causes extensive band broadening, less reproducibility and in general poorer detection limits. Significant improvements in detection limits should be achieved by incorporating $Ru(bpy)_3^{2+}$ in the mobile phase.

This paper demonstrates the feasibility of using $Ru(bpy)_3^{2+}$ ECL for detection in reversed-phase microbore LC with $Ru(bpy)_3^{2+}$ incorporated in the mobile phase. This technique is explored for the determination of erythromycin in urine and plasma as a test case. Erythromycin can be detected in these biological fluids with minimal sample preparation

due to the unique selectivity of $Ru(bpy)_3^{2+}$ CL detection.

2. Experimental

2.1. Instrumentation

Microbore LC experiments were done with the chromatographic system shown in Fig. 1. A BAS PM-80 dual-piston LC pump (Bioanalytical Systems, West Lafayette, IN, USA) was used to deliver a buffered mobile phase. A filter and a silica precolumn were used before the injection valve (Rheodyne, Berkeley, CA, USA) which was equipped with a 5 μl injection loop. The analytical column employed was a Bioanalytical Unijet C_{18}, 150×1 mm I.D., 5 μm microbore column (Bioanalytical Systems). The microbore column is the same 1/16″ outer diameter as the LC tubing which allowed it to be connected directly to the injector, completely eliminating any connecting tubing between the injector and column. Also, no tubing was used between the column and the detector. The outlet end of the column was connected directly to the electrochemical/optical ECL flow cell. The flow cell used for detection is shown in Fig. 2. The flow cell consisted of a modified BAS cross flow electrochemical cell with a stainless steel auxiliary electrode block, a PEEK working electrode block and a 16 μm Teflon spacer (Bioanalytical Systems). The auxiliary block was equipped with holes drilled and tapped for LC fittings that go directly through to the cell volume. The auxiliary block was modified to fit a 1 mm thick by 6 mm diameter glass window placed directly

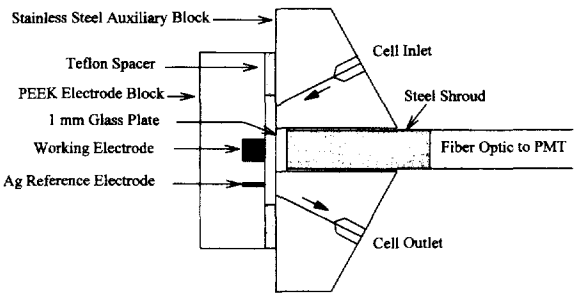

Fig. 2. Schematic diagram of the electrochemical/optical ECL flow cell.

across from the working electrode. The back of the block was drilled out to fit a fiber optic. The fiber optic touches the glass window when inserted making the distance between the fiber optic and working electrode approximately 1 mm. The working electrode block was a centered 3 mm glassy carbon electrode with a built-in Ag wire to be used as a psuedo-reference electrode. These electrodes were specially made by Bioanalytical Systems for this detection system. The working electrode was held at a potential of +1.5 V versus the Ag wire reference electrode using a CV-27 potentiostat (Bioanalytical Systems). The spacer was sandwiched between the stainless steel electrode block and the working electrode block. The stainless steel electrode block served as the auxiliary electrode. A fiber optic was used to transmit light to a Hamamatsu E990-07 end on photomultiplier tube (PMT) (Middlesex, NJ, USA), which was biased at 900 V using a high voltage power supply built in-house. Current from the PMT, ranging from 0–0.1 mA, was converted to voltage (0–1 V) using a Pacific Instruments (Con-

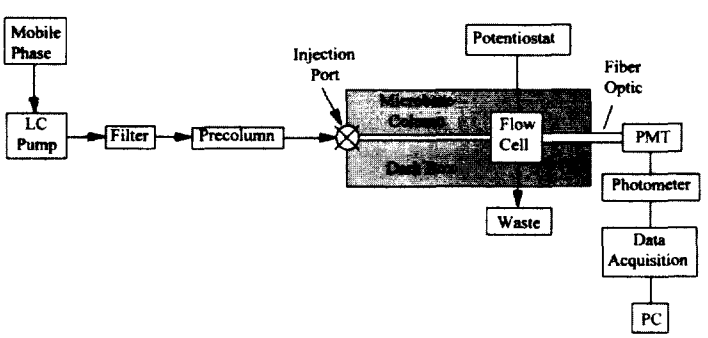

Fig. 1. Schematic diagram for the microbore LC system with chemiluminescence detection.

cord, CA, USA) Model 126 photometric amplifier. This voltage was then converted to a digital signal which was recorded using an IBM compatible PC using a BAS DA-5 data acquisition interface and ChromGraph software (Bioanalytical Systems) for data collection and analysis. This system was used for all microbore studies. Initial studies to determine the effect of adding $Ru(bpy)_3^{2+}$ to the mobile phase on the separation of erythromycin were done using a Rainin C_{18} column (100×4.6 mm I.D., 3 μm) (Woburn, MA, USA) with a Partisil ODS guard column (70×2 mm I.D.) and a SP8450 UV–Vis absorbance detector (Spectra-Physics, San Jose, CA, USA) for detection. The injection volume for these studies was 20 μl.

2.2. Reagents

Tris(2,2′-bipyridyl)ruthenium(II) chloride hexahydrate was from Aldrich (Milwaukee, WI, USA). Erythromycin was from Sigma (St. Louis, MO, USA). Acetonitrile was HPLC grade. Blood samples were obtained from Community Blood Services of Illinois (Urbana, IL, USA). The remaining sample solutions were prepared using reagent grade or better chemicals and purchased from commercial sources. All solutions were prepared in water purified using a Milli-Q water purification system (Millipore, Bedford, MA, USA).

2.3. Experimental conditions for erythromycin separations

Separation parameters for erythromycin are well documented, but previous studies in our laboratory have shown that adding $Ru(bpy)_3^{2+}$ to the mobile phase can affect retention times [19,22]. To determine what affect adding $Ru(bpy)_3^{2+}$ to the mobile phase has on the reversed-phase separation of erythromycin, an experiment was done using UV absorbance detection at 280 nm. Injections of erythromycin were made using two different mobile phases, one consisting of 100 m*M* phosphate buffer (pH 7.0)–acetonitrile (85:15, v/v) containing no $Ru(bpy)_3^{2+}$ and the same mobile phase with 25 μ*M* $Ru(bpy)_3^{2+}$ added. The flow-rate used for these experiments was 1.0 ml/min.

Separations using a microbore column with $Ru(bpy)_3^{2+}$ ECL detection were performed on erythromycin in sample matrices of water, urine and plasma. For all separations, the mobile phase employed consisted of 50 μ*M* $Ru(bpy)_3^{2+}$ in 100 m*M* phosphate buffer (pH 7.0)–acetonitrile (70:30, v/v). The mobile phase was filtered with a 0.2-μm nylon filter (Whatman, Clifton, NJ, USA) and degassed by bubbling N_2 through it prior to LC analysis. The mobile phase was pumped at 100 μl/min. All water and urine samples were filtered using a 0.2-μm syringe filter (Whatman) prior to injection to protect the column.

2.4. Sample preparation of urine and plasma samples for erythromycin analysis

Fresh urine samples were collected and diluted four-fold with Milli-Q purified water. The urine was filtered using 0.2-μm syringe filters (Whatman) before injection. Injections were made on both unspiked urine and on erythromycin-spiked urine.

Blood samples were obtained from Community Blood Services of Illinois. Ultrafiltration was done on the blood to collect protein-free plasma samples. A UF-3-12 ultrafiltration probe (Bioanalytical Systems) was used with Vacutainer tubes (Becton Dickinson, Rutherford, NJ, USA) being employed as the vacuum source. No further sample preparation was needed before injection into the LC systems. Injections were done on both the ultrafiltrate and on erythromycin spiked ultrafiltrate.

3. Results and discussion

3.1. Separation of erythromycin using LC

Initial studies were done to determine what affect adding $Ru(bpy)_3^{2+}$ to the mobile phase has on the reversed-phase separation of erythromycin. When 25 μ*M* $Ru(bpy)_3^{2+}$ is added to the mobile phase, the retention time of erythromycin increases from 80 to 350 s. This shows a significant increase in the retention time of erythromycin. By increasing the concentration of acetonitrile in the mobile phase it is possible to decrease the retention time and peak width of erythromycin. Fig. 3 shows the separation

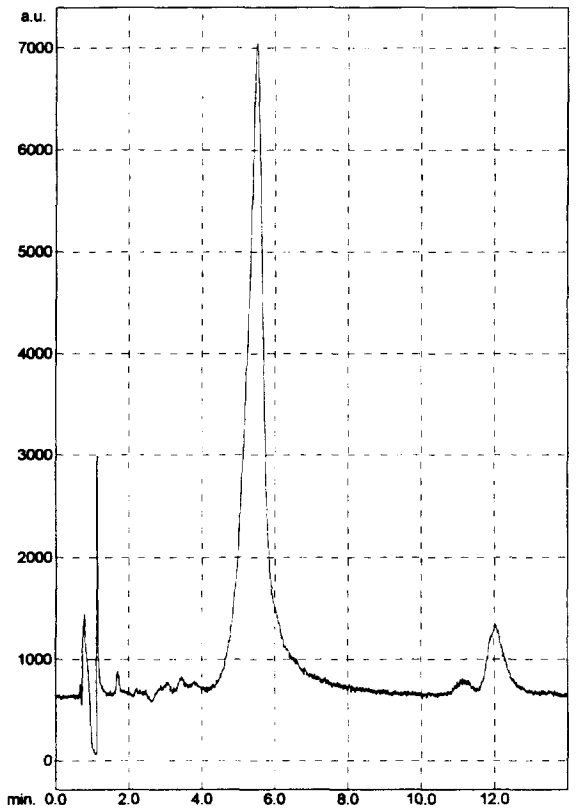

Fig. 3. Separation of 10 μ*M* erythromycin on Unijet C_{18} column 150×1 mm I.D. (5 μm) with $Ru(bpy)_3^{2+}$ ECL detection. Mobile phase consisted of 30% acetonitrile in 100 m*M* phosphate buffer at pH 7.0 with 50 μ*M* $Ru(bpy)_3^{2+}$. Flow-rate=100 μl/min, injection volume=5 μl.

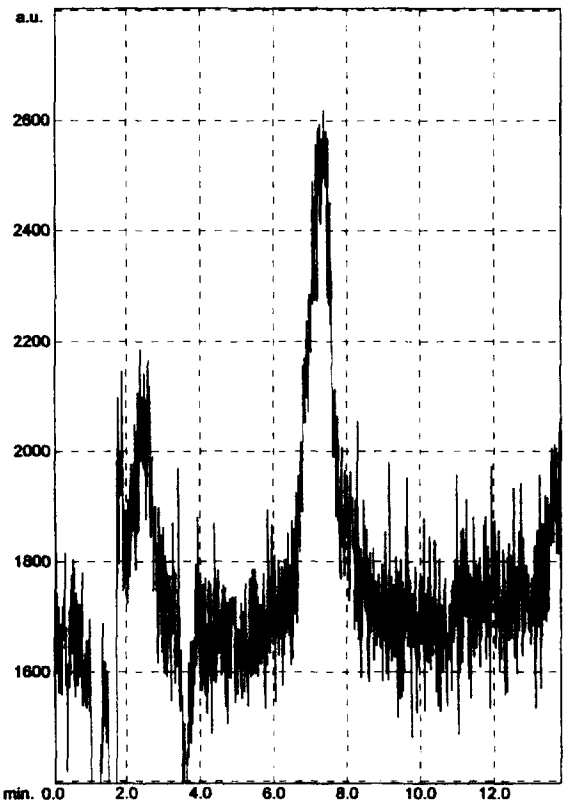

Fig. 4. Separation of 0.01 μ*M* erythromycin on Unijet C_{18} column 150×1 mm I.D. (5 μm) with $Ru(bpy)_3^{2+}$ ECL detection. Mobile phase consisted of 30% acetonitrile in 100 m*M* phosphate buffer at pH 7.0 with 50 μ*M* $Ru(bpy)_3^{2+}$. Flow-rate=100 μl/min, injection volume=5 μl.

of erythromycin with the microbore column using the conditions described in the Section 2. The *y*-axis displays the CL intensity in arbitrary units (a.u.). The retention time of erythromycin is 5.5 min. The peak width is a little larger than what is expected at this retention time using microbore LC. This is attributed to the inclusion of $Ru(bpy)_3^{2+}$ in the mobile phase. $Ru(bpy)_3^{2+}$ is retained on the stationary phase where an equilibrium is reached and the column is saturated, creating a modified stationary phase. This phenomenon has been well documented in conventional LC [19,22]. The detection limit ($S/N=3$) of erythromycin using microbore LC is 0.01 μ*M* or 50 fmol (37 pg) injected with a linear working range of four orders of magnitude. Fig. 4 shows a chromatogram of 0.01 μ*M* erythromycin injected into the microbore LC system.

3.2. Determination of erythromycin in urine and plasma

Erythromycin was determined in urine by reversed-phase microbore LC using the experimental conditions described in the Section 2. Fig. 5 shows an injection of (a) urine and (b) erythromycin-spiked urine. The first few peaks between retention times of 0.6 and 1.6 min are primarily due to the amino acids present in the urine sample which give a large CL response upon reaction with $Ru(bpy)_3^{3+}$. The erythromycin peak, which has a retention time of 5.5 min, is

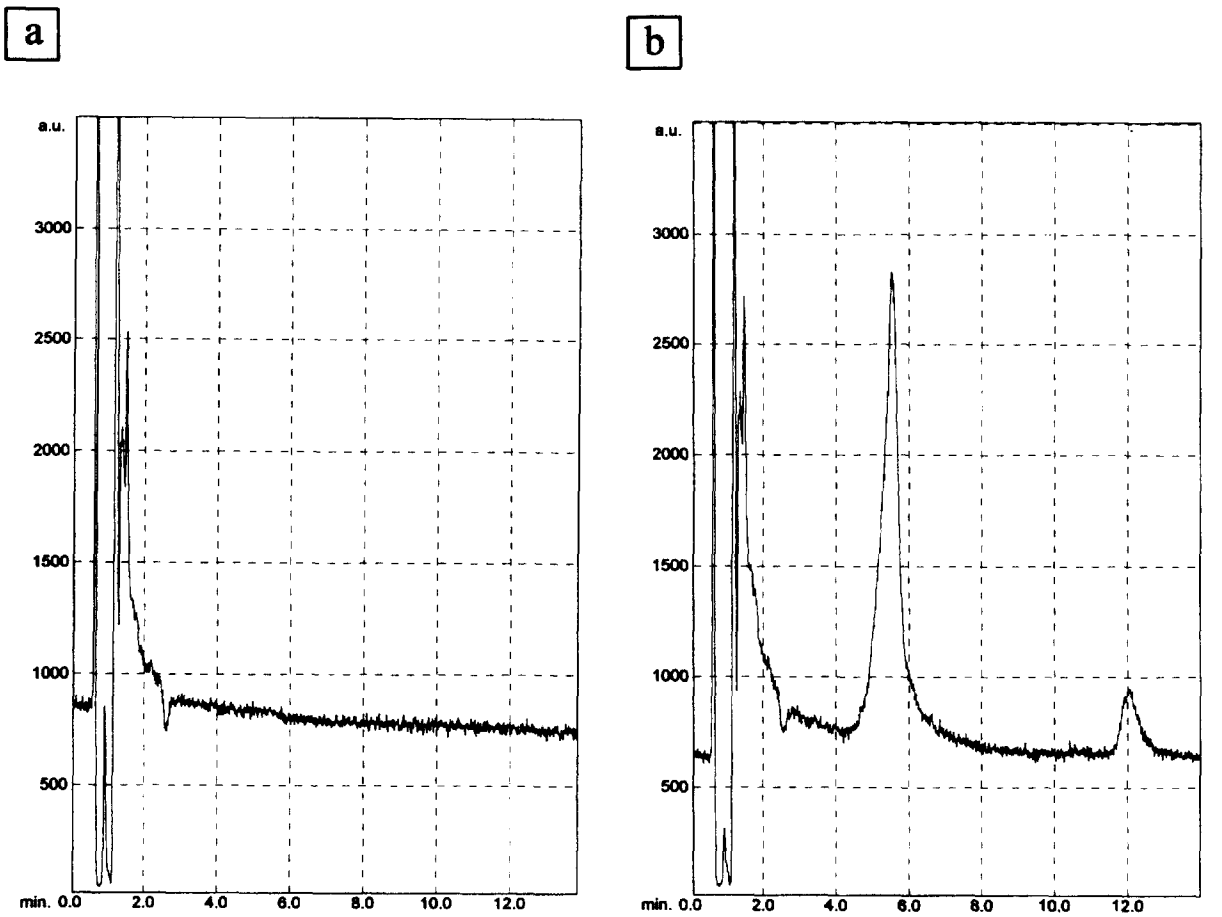

Fig. 5. Injection of (a) filtered urine sample and (b) filtered urine sample spiked with 10 μ*M* erythromycin on Unijet C_{18} column 150×1 mm I.D. (5 μm) with $Ru(bpy)_3^{2+}$ ECL detection. Mobile phase consisted of 30% acetonitrile in 100 m*M* phosphate buffer at pH 7.0 with 50 μ*M* $Ru(bpy)_3^{2+}$. Flow-rate = 100 μl/min, injection volume = 5 μl.

well resolved from these first peaks. The detection limit for erythromycin in urine was determined to be 0.05 μ*M*.

Erythromycin was also determined in plasma samples prepared using the method described previously. Chromatograms of (a) plasma and (b) erythromycin-spiked plasma are shown in Fig. 6. Again, the first peaks between retention times of 0.6 and 1.5 min are primarily due to amino acids present in plasma. Erythromycin is cleanly resolved from these peaks at a retention time of 5.5 min. The detection limit of erythromycin in plasma is below 0.1 μ*M*.

For both the urine samples and plasma samples very little sample preparation is needed. This is because $Ru(bpy)_3^{2+}$ CL is a very selective detection method which makes it useful for detection of compounds in complex biological samples. The majority of the compounds present in these biological fluids do not give light with $Ru(bpy)_3^{3+}$, which simplifies the separation greatly. For the determination of erythromycin in biological samples using electrochemical or UV absorbance detection, additional sample preparation is necessary. An extraction is almost always done on plasma samples before injection to decrease the number of interfering compounds. This adds both time and cost to the analysis. The ability to analyze erythromycin using microbore LC is also a benefit. Very small sample

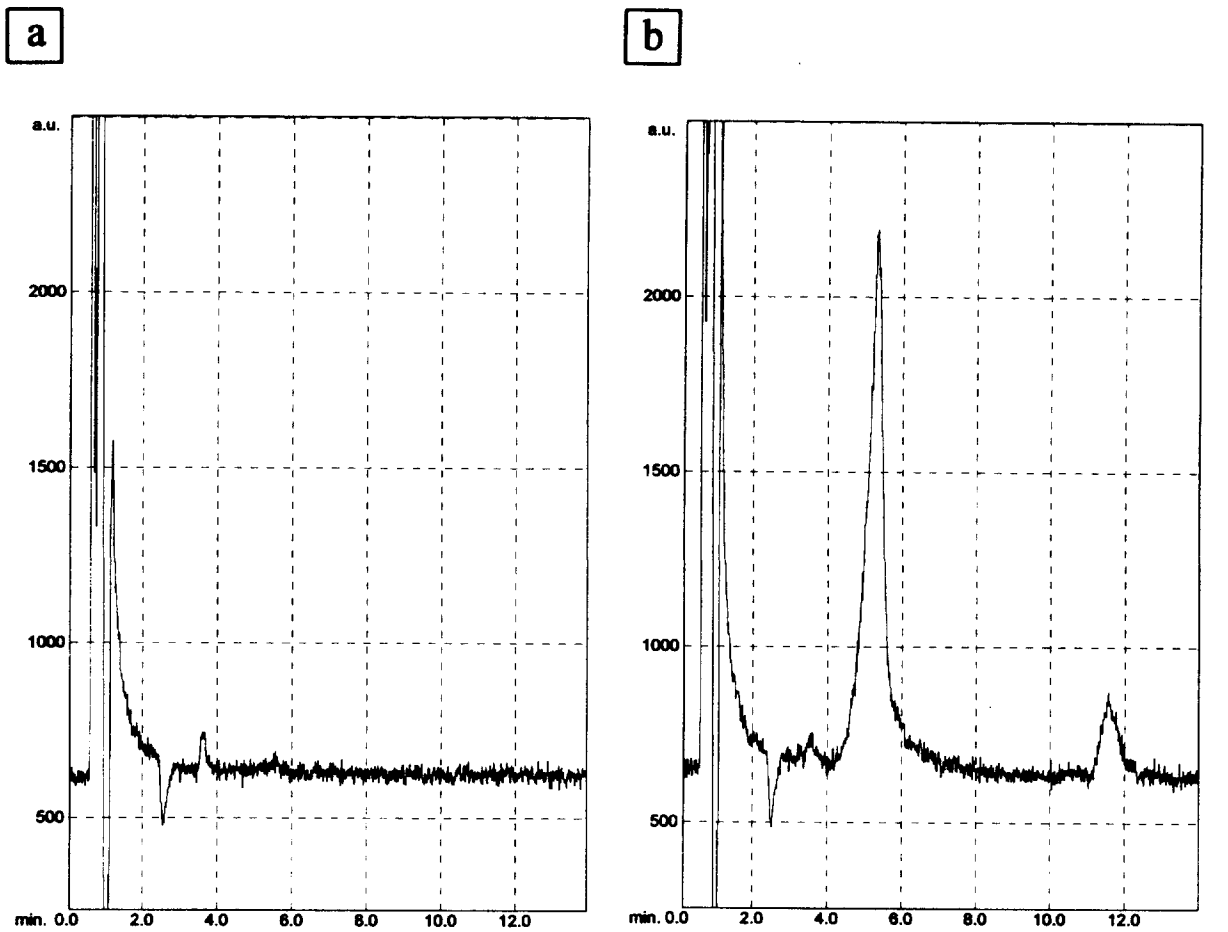

Fig. 6. Injection of (a) blood ultrafiltrate sample and (b) blood ultrafiltrate sample spiked with 10 μ*M* erythromycin on Unijet C_{18} column 150×1 mm I.D. (5 μm) with $Ru(bpy)_3^{2+}$ ECL detection. Mobile phase consisted of 30% acetonitrile in 100 m*M* phosphate buffer at pH 7.0 with 50 μ*M* $Ru(bpy)_3^{2+}$. Flow-rate = 100 μl/min, injection volume = 5 μl.

sizes are required, which has proven to be important in biological and clinical work. Sampling is sometimes done using in vivo techniques such as microdialysis and ultrafiltration. Being able to inject small volumes allows for more frequent sampling. The possibility exists of coupling electrochemical or UV absorbance detection to microbore LC for the detection of erythromycin, but the fluorescence detection of erythromycin is not very feasible for use with microbore LC. For fluorescence detection, both a derivatization and an extraction are done post-column [8] which leads to band broadening and sample dilution.

4. Conclusions

$Ru(bpy)_3^{2+}$ ECL coupled with microbore LC is a sensitive detection method for the determination of erythromycin in urine and plasma samples. This detection system offers the advantage of minimal sample preparation over the commonly used detection methods with comparable or better detection limits. The previously reported techniques require an extraction step for the determination of erythromycin in biological samples. Some techniques also require a derivatization. With $Ru(bpy)_3^{2+}$ ECL detection, the only sample preparation required is filtering for urine

samples and using ultrafiltration on plasma samples for protein removal. The detection limits obtained for erythromycin in urine and plasma are 0.05 μM (0.25 pmol injected) and 0.1 μM (0.5 pmol injected), respectively, at a S/N of 3. This is a better detection limit than can be obtained using UV absorbance detection. Lower concentration detection limits have been reported with electrochemical and fluorescence detection, but when sample size is considered, $Ru(bpy)_3^{2+}$ ECL provides better sensitivity. This is considering both the sample size injected and the amount of sample necessary for sample preparation. This chromatographic system is useful for the sensitive determination of erythromycin and can be applied to other compounds detectable by $Ru(bpy)_3^{2+}$ ECL in both biological and nonbiological samples.

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