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Voltammetric investigation of macrolides by an HPLC-coulometric assay

Yong-Hak Kim, Jairaj V. Pothuluri, Carl E. Cerniglia *

Division of Microbiology, National Center for Toxicological Research, U.S. Food and Drug Administration, 3900 NCTR Road, Jefferson, AR 72079, USA

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Abstract

Voltammograms of macrolides, including anhydroerythromycin A, azithromycin, erythromycin A, erythromycin A enol ether, pseudoerythromycin A enol ether, oleandomycin and tylosin have been investigated using a dual electrode cell in combination with a high-throughput LC method. The half-wave potentials ($E_{1/2}$) of the seven macrolides investigated ranged from 0.734 to 0.866 V, and the current responses reached the maxima at over 1.0 V. The current response of the downstream electrode displayed a non-linear behavior at high potentials over +0.75 V, probably because of polarization of solvent components, e.g., water. The HPLC-coulometric assay was optimized with the potentials of the upstream and downstream electrodes at +0.65 and +0.85 V, respectively. This method is suitable for detection of 14- and 15-membered macrolides (sensitivity < 0.05 μg ml^{-1}), but not for a 16-membered macrolide, tylosin (sensitivity > 0.1 μg ml^{-1}). The assay shows interferences from biomatrices in rat's blood plasma and serum, and human urine, but they were effectively removed by a cold acetonitrile extraction method.

Keywords: Coulometric assay; HPLC; Macrolides; Voltammogram

1. Introduction

Macrolides consist of a 12- to 16-membered ketolide ring, to which one or more sugar moieties are substituted. This class of antibiotics has been widely used in humans and food-producing animals. Because of extensive worldwide use, human clinical and veterinary macrolide antibiotics can be widely distributed in biological and environmental samples. Antibiotics released into the environment are of concern because they have potential to accelerate development of antibiotic resistance in bacteria from food-producing animals and resistance gene transfer to humans [1]. Foods and environments contaminated with antibiotics could function as reservoirs of antibiotic resistance genes. Particularly, erythromycin strongly inhibits the microalga, *Selenastrum capricornutum* (EC_{50} = 0.037 mg l^{-1}; ref. [2]). Therefore, it is necessary to develop an accurate and robust detection method for monitoring biological and environmental exposure to macrolide antibiotics.

Biological assays have been introduced to determine the concentration of a single type of macrolide using erythromycin-sensitive strains as indicators [3,4]. This method is routinely used for the determination of the efficacy and the susceptibility of antibiotics toward test microorganisms. The biological activities of antibiotics are semi-quantitatively measured, typically using twofold serial dilutions of drug concentrations in microtiter plates. Although it is convenient for the estimation of effective drug concentrations by logit analysis, it is difficult to distinguish activities of individual drugs in mixtures showing synergistic effects [2]. Most drawbacks arise from test accuracy and precision, because the bioassay is largely dependent on culture techniques (e.g., liquid culture and plate culture) and the physiological states of indicator microorganisms.

* Corresponding author. Tel.: +1 870 543 7341; fax: +1 870 543 7307.
E-mail address: ccerniglia@nctr.fda.gov (C.E. Cerniglia).

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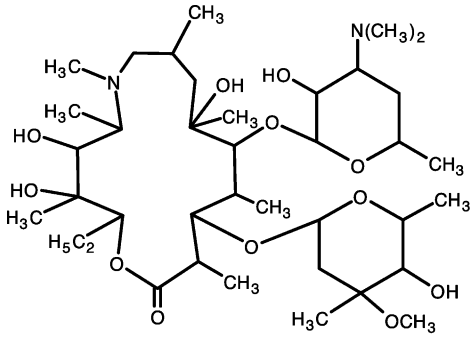
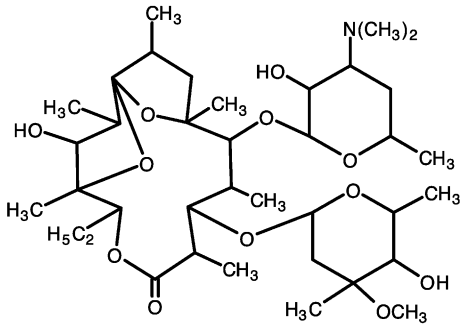
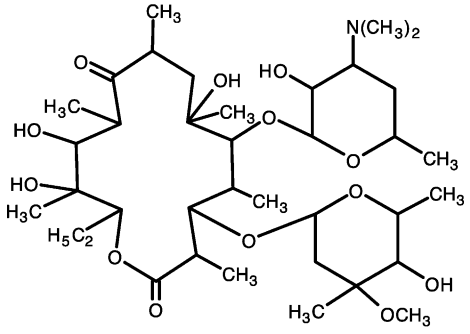


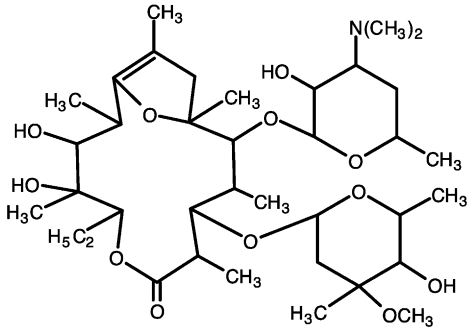
AZT: azithromycin



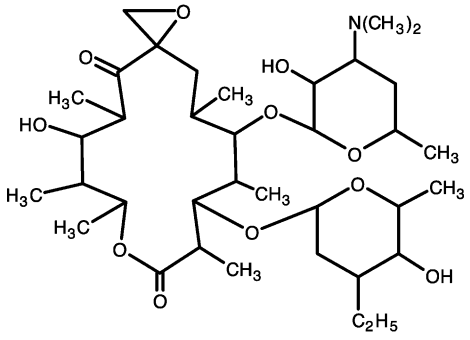
AEA: anhydroerythromycin A



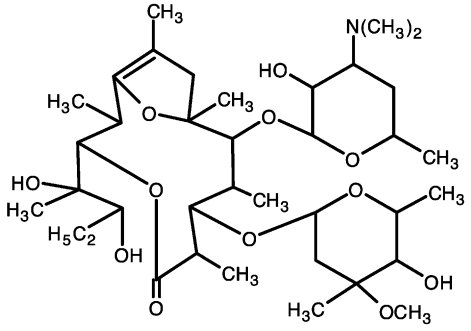
EA: erythromycin A



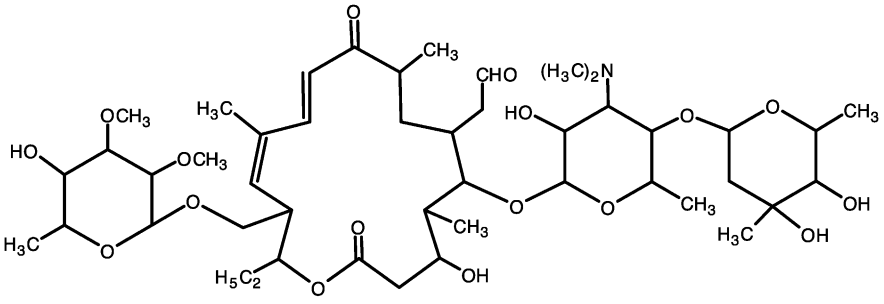
EMEN: erythromycin A enol ether



OM: oleandomycin



psEMEN: pseudoerythromycin A enol ether



TYL: tylosin

Fig. 1. Structures of macrolides.

UV detection in concert with high-performance liquid chromatography (HPLC) has been conventionally used for the determination of macrolides in drugs, blood plasma and serum, since introduced by Tsuji and Groetz [5]. Particularly, this method is suitable for sensitive detection of 16-membered macrolides with conjugated double bonds, which have strong UV absorption at about 230 nm [6].

Alternatively, fluorescence detection has been employed for sensitive detection of fluorescently labelled erythromycin at less than 0.01 $\mu g\,ml^{-1}$ in serum [7,8]. Generally, HPLC analysis with fluorescence detection requires complex procedures for sample extraction, reconstitution with appropriate buffers for HPLC analysis, and post-column derivatization of macrolide molecules with proper fluorescent dyes.

Liquid chromatography combined with mass spectrometry (LC–MS or LC–MS/MS) has been employed for the sensitive detection of macrolides [9–11]. LC/electrospray ionization (ESI) mass spectrometry with collision-induced dissociation (CID) is considered to be a powerful tool for the identification of compounds. However, it is difficult for the acquisition of mass spectral data from some molecules that do not respond to ESI mass analysis. The observed mass spectra of analytes can vary because the composition of the HPLC mobile phase has a significant effect on electrochemical processes for the ESI ion source. Optimization of LC/ESI mass spectrometry is time-consuming for high-throughput analyses of macrolides in biological and environmental samples.

Electrochemical detection, using amperometric and coulometric detectors, is the standard method for the sensitive detection of macrolides, because most biologically active macrolide antibiotics contain electrochemically sensitive tertiary amines [12–22]. The use of a dual electrode cell is beneficial for improving the selectivity of electrochemical detection of macrolides between the two potentials of the upstream (or screening) and downstream (or working) electrodes. In the oxidative mode, the potential of the upstream electrode should be lower than that of the downstream electrode to minimize the electrochemical oxidation of the HPLC solvent components and impurities at the downstream electrode. We recently used an HPLC-coulometric method for the determination of erythromycin A and its degradation products, including erythromycin A-1-carboxylic acid from the degradation by erythromycin esterase [23,24], anhydroerythromycin A, erythromycin A enol ether, pseudoerythromycin A enol ether, and others produced from chemical degradation by acid/base-catalyzed reactions in aqueous solutions [25], and clay-catalyzed reactions in homoionic clays [26]. However, little is known about the current responses of various macrolides to optimize electrochemical detection.

The objective of this study was to investigate the current responses of various macrolides containing electrochemically active tertiary amines (Fig. 1). We propose an HPLC-coulometric method suitable for the robust and high-throughput analysis of azithromycin, erythromycin, oleandomycin, tylosin, and their degradation products. The potentials of the upstream and downstream electrodes of a dual electrode cell were optimized for the selective and sensitive detection of these macrolides. The method was validated by analyses of individual macrolides and mixtures added to rat's blood plasma, serum and human urine.

2. Materials and methods

2.1. Chemicals

Erythromycin hydrate and 2-hydroxycarbazole were purchased from Sigma–Aldrich Chemical Co., St. Louis, MO. Oleandomycin (phosphate salt) and tylosin were obtained from ICN Pharmaceuticals, Inc., Costa Mesa, CA. Azithromycin was purified from tablets of Zithromax™ (Pfizer, Inc., New York, NY). Anhydroerythromycin A, erythromycin enol ether, and pseudoerythromycin A enol ether were prepared as described by Kim et al. [25]. Macrolides were recrystallized in aqueous methanol unless the purity exceeded 95% by LC/ESI mass analysis [25]. All other chemicals and solvents used were of analytical grade (J.T. Baker, Philipsburg, NJ), and water was deionized using a Millipore Super-Q Plus water purification system (Bedford, MA).

2.2. Voltammograms

Each 1 $\mu g\,ml^{-1}$ macrolide standard was prepared in ammonium acetate (pH 7.0; 0.25 M)-acetonitrile-methanol (40:50:10, v/v/v). The voltammograms were examined using an ESA Coulochem® II-500 electrochemical detector (ESA, Inc., Chelmsford, MA), as the potential of the downstream electrode (E_2) varied from 0 to 1 V with the potential of the upstream electrode (E_1) at 0 V. The current response to macrolides varied with changes in the potential of the downstream electrode.

Theoretically, the coulometric assay requires no preliminary calibration against standards, because the current response is fundamentally related to the concentration of analyte, A, according to Faraday's law. The current, i, is proportional to the concentration gradient of analyte between the bulk of the solution, c_A, and the thin layer of the electrode surface, c_A^0:

$$i = k_A(c_A - c_A^0) \tag{1}$$

where k_A is a constant derived from Faraday's law. The current increases, as c_A^0 decreases with increasing electrode potential. The maximum, i_{max}, is reached, when the surface concentration of analyte approaches zero ($c_A^0 \rightarrow 0$).

$$i_{max} = k_A c_A \tag{2}$$

Subtracting Eq. (2) from Eq. (1) gives

$$c_A^0 = \frac{i_{max} - i}{k_A} \tag{3}$$

Similarly, the current, i, is expressed by the concentration gradient of product, P, between the bulk of the solution, c_P, and the thin layer of the electrode surface, c_p^0:

$$i = -k_P(c_P - c_P^0) \quad (4)$$

in which the minus sign means negativity of the concentration gradient of the product. The concentration of product in the bulk of the solution is negligible because of electrolysis ($c_P \to 0$). Thus,

$$c_P^0 = \frac{i}{k_P} \quad (5)$$

Following Nernst's equation, the relationship between the current (μA) and the applied electrode potential, E_{appl} (V), is expressed in the steady state:

$$E_{appl} = E_A^0 + \frac{0.0592}{n} \ln \frac{c_P^0}{c_A^0} = E_A^0 + \frac{0.0592}{n} \ln \frac{k_A}{k_P} + \frac{0.0592}{n} \ln \frac{i}{i_{max} - i} \quad (6)$$

where E_A^0 is the standard oxidation potential at 25 °C and 1 atm, and n is the number of electrons transferred from the electrode per mole of analyte ($n = 1$ for all macrolides containing one tertiary amine group). When $i = 1/2\, i_{max}$, the third term on the right side of Eq. (6) is equal to zero. At this point, the E_{appl} is defined as the half-wave potential, $E_{1/2}$.

$$E_{1/2} = E_A^0 + 0.0592 \cdot \ln \frac{k_A}{k_P} \quad (7)$$

Substituting this expression into Eq. (6),

$$E_{appl} = E_{1/2} + 0.0592 \cdot \ln \frac{i}{i_{max} - i} \quad (8)$$

Solving this equation for the current response (i) provides a logistic equation for the curve-fitting of the sigmoid voltammogram with variable E_{appl}.

$$i = \frac{i_{max}}{1 + e^{(E_{1/2} - E_{appl})/0.0592}} \quad (9)$$

The maximum current response (i_{max}) and the half-wave potential, $E_{1/2}$, were estimated by the least square method.

2.3. Liquid chromatography

Concentrations of macrolides were determined by reversed-phase HPLC with electrochemical detection. The chromatographic system consisted of an ESA solvent delivery module 581 (ESA, Inc., Chelmsford, MA), a guard column packed with C_{18}-μBondapak (Alltech Associates Inc., Deerfield, IL) and a reversed-phase Radial-Pak Resolve Silica cartridge (5 μm particle size, 0.8 cm × 10 cm, Waters Co., Milford, MA) fixed with a Waters Radial Compression Module. An ESA Coulochem® II-500 electrochemical detector was connected with an ESA Model 5020 guard cell and an ESA Model 5010 dual electrode cell. The potential of the guard cell was set at +0.90 V, and the potentials of the upstream and downstream electrodes in the dual cell were set at +0.65 and +0.85 V, respectively. Samples were injected using an ESA Model 545 Autosampler installed with a Rheodyne 7125 injector and a 200 μl loop. The mobile phase consisted of ammonium acetate (pH 7.0; 0.25 M)-acetonitrile-methanol (40:50:10, v/v/v) at 1 ml min^{-1}.

2.4. Validation tests

Blood plasma and serum were prepared from blood samples obtained from four Fischer 344 inbred rats (NIH, Washington, DC) and four Crl:COBS CD(SD) BR outbred rats (Charles River, Wilmington, MA). Urine was provided by a volunteer who had not been medicated with antibiotics for at least 2 years before the experiments. Individual macrolide compounds and the mixture were added to blood plasma, serum and urine in a range of 0.05–125 μg ml^{-1}, to which 2-hydroxycarbazole (final concentration, 2 μg ml^{-1}) was added as an internal standard. After 1 h of incubation at 37 °C on a rotary shaker (200 rpm), the samples were thoroughly mixed with one tenth of the volume of 3.4 M ammonium sulfate solution (final concentration, 0.31 M) and three volumes of ice-cold acetonitrile for 30 min, and were placed at −20 °C for 1 h. After centrifugation for 20 min at 14,000 rpm using an Eppendorf® microcentrifuge (Brinkmann Instruments, Inc., Westbury, NY), the supernatants were gently transferred to vials for analysis by HPLC with electrochemical detection. Every test was performed independently in quadruplicate. The accuracy and precision of tests are reported as means and standard errors of the means.

3. Results and discussion

3.1. Voltammograms

As the applied potential of the downstream electrode varied from 0 to +1.0 V, the current responses to macrolide molecules exhibited sigmoid curves. The observed current response was plotted against the applied potential at 20-mV intervals. Fig. 2 shows plots of the current response versus the applied potential, which fitted well to the logistic Eq. (9) ($R^2 > 0.99$ for all). From the voltammograms, the half-wave potentials ($E_{1/2}$) of anhydroerythromycin A, azithromycin, erythromycin A, erythromycin A enol ether, oleandomycin, pseudoerythromycin A enol ether and tylosin were estimated at 0.837, 0.827, 0.866, 0.817, 0.812, 0.792 and 0.734 V, respectively, and the maximum current responses towards each 1 μg ml^{-1} of the standards reached 3.27, 11.9, 5.01, 10.4, 2.51, 4.01 and 2.75 μA (Table 1). The maximum current responses were mostly observed at over +1.0 V, consistent with the other voltammetric studies using amperometric and coulometric detectors [12,15,16]. The mean applied potential for the 5% current response to the macrolides investigated

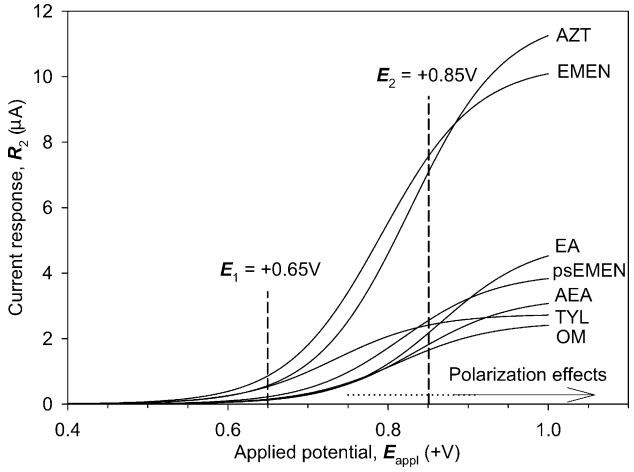


Fig. 2. Voltammograms of macrolide standards (each 1 μg ml^{-1}) in the oxidative mode. Abbreviations are used for anhydroerythromycin A (AEA), azithromycin (AZT), erythromycin A (EA), erythromycin A enol ether (EMEN), oleandomycin (OM), pseudoerythromycin A enol ether (psEMEN) and tylosin (TYL).

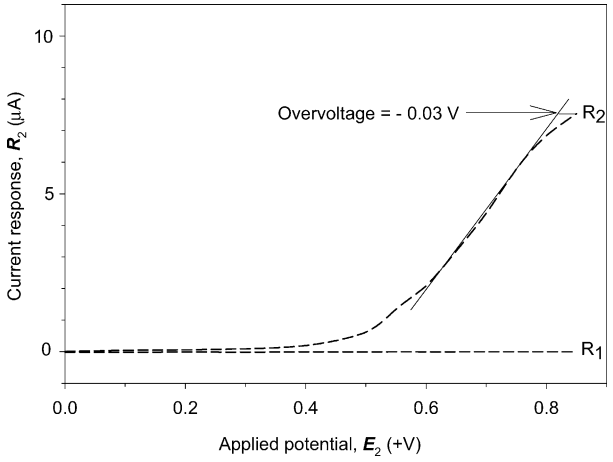


Fig. 3. The current responses of the upstream (R_1) and downstream (R_2) electrodes in a dual electrode cell to ammonium acetate (pH 7.0; 0.25 M)-acetonitrile-methanol (40:50:10, v/v/v). The potential of the downstream electrode (E_2) varies from 0 to +0.85 V with the potential of the upstream electrode (E_1) at 0 V.

was +0.638 V, and the mean for the 95% current response was +0.986 V.

The current response (R_2) at the downstream electrode of the dual electrode cell showed a non-linear behavior at over +0.75 V, because of the polarization of the solvent components, e.g., water (Fig. 3). From the curvature, the overvoltage required to achieve 7.5 μA was measured at −0.03 V. In contrast, the polarization of the downstream electrode did not influence the current response (R_1) at the upstream electrode, probably because a constant flow prevented polarization effects of the reactant species on the surface of the upstream electrode. If samples were consecutively analyzed at a large applied potential > +0.87 causing the polarization of water, the kinetic polarization might gradually arise from poisoning on the surface of the downstream electrode [13,14]. This effect will be much greater when samples include metallic impurities, such as copper, lead, zinc and mercury [27]. To reduce undesired polarization effects of solvent components and trace metallic impurities, the applied potential should be retained at less than +0.87 V.

Table 1
Voltammetric parameters of macrolides

Macrolides[a]	Voltammetric parameters		E_{appl} (V)[d]	
	i_{max} (μA)[b]	$E_{1/2}$ (V)[c]	5% i_{max}	95% i_{max}
AEA	3.27 (0.20)	0.837 (0.006)	0.663	1.01
AZT	11.9 (0.74)	0.827 (0.007)	0.653	1.00
EA	5.01 (0.60)	0.866 (0.011)	0.692	1.04
EMEN	10.4 (0.79)	0.792 (0.010)	0.618	0.966
OM	2.51 (0.33)	0.812 (0.016)	0.638	0.986
psEMEN	4.01 (0.61)	0.817 (0.018)	0.643	0.991
TYL	2.75 (0.14)	0.734 (0.009)	0.560	0.908

[a] Abbreviations used: AEA, anhydroerythromycin A; AZT, azithromycin; EA, erythromycin A; EMEN, erythromycin A enol ether; OM, oleandomycin; psEMEN, pseudoerythromycin A enol ether; TYL, tylosin.
[b] i_{max}, the maximum current response (μA) and the standard error of estimate in parentheses.
[c] $E_{1/2}$, the half-wave potential (V) and the standard error of estimate in parentheses.
[d] The applied potentials (V) for the 5 and 95% current responses to macrolides are calculated by Eq. (8), when i is equal to 0.05i_{max} and 0.95i_{max}, respectively.

3.2. HPLC-coulometric assay of macrolides

When the potentials of the upstream and downstream electrodes were set at +0.65 and +0.85 V, respectively, the current response resulted in good linearity in a range of 0.05–50 μg ml^{-1} (r > 0.999 for all). With a sensitivity range (R_2) of 10 μA, the minimum detection limits of macrolides investigated ranged from 0.022 to 0.104 μg ml^{-1} at a signal-to-noise ratio of three (Table 2). The HPLC system used

Table 2
HPLC-coulometric assays of macrolides[a]

Macrolides[b]	Retention time (min)	A[c] (μA/μg ml^{-1})	MDL[d] (μg ml^{-1})
AEA	6.30 (0.009)	1.23 (0.001)	0.041
AZT	9.27 (0.056)	1.09 (0.029)	0.046
EA	5.52 (0.012)	1.47 (0.003)	0.034
EMEN	10.61 (0.032)	1.97 (0.005)	0.025
OM	4.46 (0.004)	1.81 (0.004)	0.028
psEMEN	7.88 (0.018)	1.25 (0.002)	0.040
TYL	4.25 (0.003)	0.48 (0.001)	0.104

[a] Analytical conditions: potentials of the guard cell and the upstream and downstream electrodes, +0.9, +0.65 and +0.85 V, respectively; current offset of the downstream electrode, 1 μA; injection volume, 200 μl; mobile phase, ammonium acetate (pH 7.0; 0.25 M)-acetonitrile-methanol (40:50:10, v/v/v); flow rate, 1 ml min^{-1}.
[b] Abbreviations used in Table 1.
[c] A, the specific current response (μA/μg ml^{-1}).
[d] The minimum detection limit at the current response of 0.05 μA.

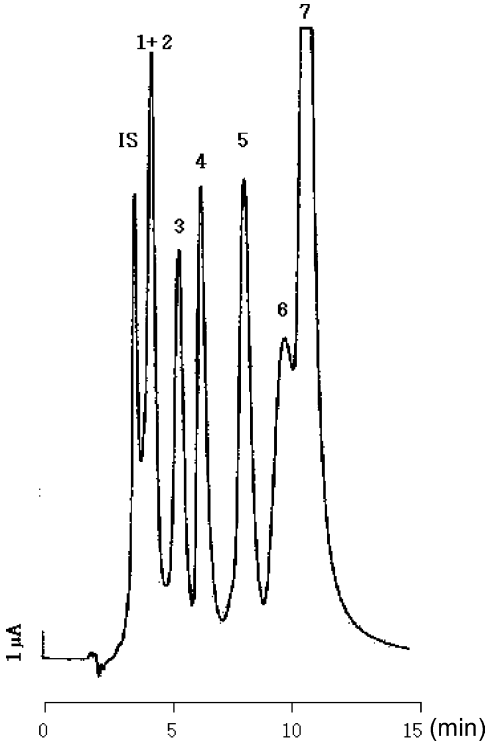


Fig. 4. Chromatogram for each 50 μg ml^{-1} of (1) oleandomycin, (2) tylosin, (3) erythromycin A, (4) anhydroerythromycin A, (5) pseudoerythromycin A enol ether, (6) azithromycin, (7) erythromycin A enol ether, and (IS) internal standard, 2-hydroxycarbazole (2 μg ml^{-1}). The assay conditions follow: injection volume, 200 μl; mobile phase, ammonium acetate (pH 7.0; 0.25 M)-acetonitrile-methanol delivered at 1 ml min^{-1}; potentials of the guard cell and the upstream and downstream electrodes, +0.90, +0.65 and +0.85 V, respectively.

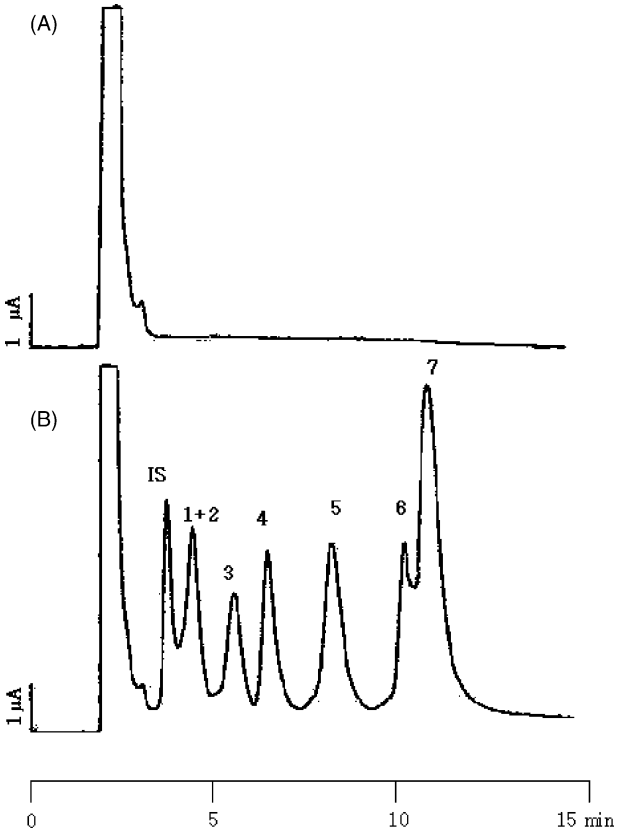


Fig. 5. Chromatograms of the acetonitrile extraction of (A) blank urine and (B) urine spiked with 50 μg ml^{-1} of each macrolide and 2 μg ml^{-1} of 2-hydroxycarbazole (IS). The assay conditions and abbreviations are given in Fig. 4.

appeared to be suitable for the separation of individual compounds in the mixture, except for tylosin, which overlapped with oleandomycin (Fig. 4). With consecutive injections of the mixture, one cycle of the HPLC-coulometric analysis was completed within 15 min. This method appeared to be suitable for the selective and sensitive detection of 14- and 15-membered macrolides (sensitivity < 0.05 μg ml^{-1}), but not for a 16-membered macrolide, tylosin (sensitivity > 0.1 μg ml^{-1}).

3.3. Determination of macrolides in biological fluids

Macrolides consist of neutral and/or amino sugar(s) and a macrocyclic lactone ring with hydrophilic and hydrophobic characters. The structural characters of macrolides influence not only the physiological behavior [28] but also the physicochemical properties in aquatic and soil environments [25,26]. Liquid–liquid extraction with an organic solvent (e.g., chloroform, dichloromethane, ethyl acetate and *tert*-butyl methyl ether) or solid-phase extraction with a hydrophobic resin necessitates time-consuming sample preparation and reconstitution procedures using appropriate pH buffers before HPLC analysis [29,30]. In contrast, the acetonitrile extraction method used in this study required neither sample clean-up nor reconstitution.

Acetonitrile extraction showed no significant interference of the HPLC-coulometric assay from biomatrices in rat's blood plasma, serum and human urine (Fig. 5). When the standard curves of individual macrolides added to the biological fluids were constructed with at least five data points in a range of 0.5–125 μg ml^{-1}, the extracted amounts showed good linearity ($r > 0.99$ for all) and high reproducibility (coefficient of variation < 10% for all). The internal standard, 2-hydroxycarbazole (2 μg ml^{-1}), was extracted nearly up to 100%. The percent extraction efficiency of the single extraction varied from compound to compound with the average of 80% in a range of 30–108% (Table 3). Except for erythromycin A added to blood serum (showing 30% recovery), the cold acetonitrile extraction method was simple and practical for the determination of concentrations of macrolides in biological fluids, using external standard curves. It seemed that extraction efficiency was positively related to the compound's hydrophobicity (i.e., retention time) under HPLC conditions. Additionally, the extraction efficiency might be affected by charge interactions of macrolides with biomatrices, as observed with phospholipids [28].

Table 3
Extraction of macrolides from biological fluids with cold acetonitrile

Macrolides[a]	Plasma		Serum		Urine	
	A[b]	%Recovery[c]	*A*	%Recovery	A	%Recovery
2-OHC	n.d.[d]	102	n.d.[d]	98	n.d.[d]	110
AEA	1.33 (0.008)	108	1.27 (0.005)	103	1.05 (0.002)	85
AZT	0.75 (0.004)	69	0.77 (0.009)	71	0.81 (0.057)	74
EA	1.00 (0.008)	68	0.44 (0.001)	30	1.06 (0.007)	72
EMEN	2.07 (0.013)	105	1.97 (0.031)	100	1.54 (0.087)	78
OM	1.29 (0.034)	71	1.21 (0.005)	67	1.43 (0.006)	79
psEMEN	1.06 (0.017)	85	1.01 (0.009)	81	1.08 (0.103)	86
TYL	0.41 (0.015)	85	0.34 (0.020)	70	0.40 (0.011)	84

[a] Abbreviations of macrolides used in Table 1; 2-OHC, 2-hydroxycarbazole.
[b] A, specific current response ($\mu A/\mu g\,ml^{-1}$).
[c] Percent recovery in the acetonitrile extraction is calculated from the ratio of the specific current response to the corresponding value given in Table 2.
[d] n.d., not determined.

4. Conclusions

Electrochemical detection was suitable for selective and sensitive detection of macrolide antibiotics containing tertiary amine groups. The coulometric assay using a dual electrode cell was optimized with the potentials of the upstream and downstream electrodes at +0.65 and +0.85 V, respectively. A reversed-phase HPLC method with electrochemical detection was suitable for the determination of concentrations of 14- and 15-membered macrolides, including azithromycin, erythromycin A, oleandomycin and erythromycin A derivatives with high sensitivity $< 0.05\,\mu g\,ml^{-1}$, but not for a 16-membered macrolide, tylosin. The cold acetonitrile extraction was simple and practical for a robust and high-throughput HPLC-coulometric assay of macrolides in biological fluids.

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