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High-performance liquid chromatographic assay with electrochemical detection for azithromycin in serum and tissues

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ABSTRACT

High-performance liquid chromatographic methods using reversed-phase chromatography and electrochemical detection have been developed for the quantitation of azithromycin in serum and tissues of laboratory animals and humans. Serum sample preparation involved addition of internal standard, alkalinization, and solvent extraction. Tissue sample preparation involved Polytron homogenization in acetonitrile containing internal standard, evaporation of the supernatant, alkalinization of the residue, and solvent extraction. Serum samples were chromatographed on an alkylphenyl-bonded silica column eluted with pH 6.8–7.2 mobile phase with a dual-electrode coulometric detector operated in the oxidative screen mode. Serum and tissue samples were chromatographed on a γRP-1 alumina column with pH 11 mobile phase with a glassy carbon amperometric detector. Recovery of azithromycin was 87% from serum and 85% from tissues. Linear standard curves were prepared in serum over two concentration ranges (0.01–0.20 and 0.20–2.0 μg/ml) and in tissues over several concentration ranges (0.1–2, 1–10, 10–100, and 100–1000 μg/g). In serum and tissues, intra- and inter-assay precision ranged from 1 to 8% and 4 to 11%, respectively. The tissue assay has been applied to liver, kidney, lung, spleen, muscle, fat, brain, tonsil, lymph nodes, eye, prostate and other urological tissues, and gynecological tissues.

INTRODUCTION

Azithromycin is the first member of a class of macrolide antibiotics called azalides [1]. It is an effective therapeutic agent for oral treatment of sexually transmitted diseases, upper and lower respiratory tract infections, and skin and skin structure infections. Azithromycin (I, Fig. 1) differs structurally from erythromycin by the insertion of a methyl-substituted nitrogen at position 9a in the lactone ring to create a fifteen-membered macrolide. This modification produces an enhanced spectrum and potency against bacteria compared with erythromycin [2] and superior stability in an acid environment [3]. Azithromycin also has greater oral bioavailability, much longer elimination half-lives, and much higher tissue concentrations than erythromycin in animals [4,5] and in humans [6]. The *in vivo* efficacy of azithromycin is attributed to its high and sustained tissue concen-

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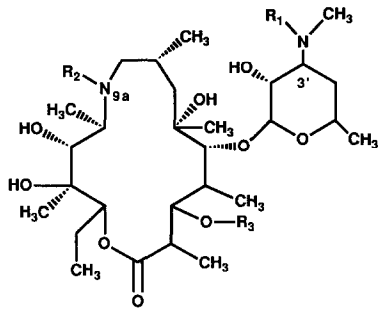

Compound	R_1	R_2	R_3
I	CH_3	CH_3	Cladinose
II	CH_3	$CH_2CH_2CH_3$	Cladinose
III	CH_3	CH_2CCH	Cladinose
IV	CH_3	H	Cladinose
V	H	CH_3	Cladinose
VI	CH_3	CH_3	H

Fig. 1. Structures of azithromycin (I), assay internal standards II and III, and potential azithromycin metabolites 9a-N-desmethylazithromycin (IV), 3'-N- desmethylazithromycin (V), and descladinoseazithromycin (VI).

trations [7,8]. To characterize the pharmacokinetics of azithromycin, assays for the determination of azithromycin in serum and tissues were developed.

As with erythromycin, azithromycin can be quantitated by microbiological assay [4]. Although bioassays have appropriate detection limits, they lack specificity due to the possible presence of active metabolites or other antibiotics. One possible metabolite of azithromycin, 9a-N-desmethylazithromycin (IV, Fig. 1), is active against many strains of bacteria. High-performance liquid chromatographic (HPLC) assays for erythromycin in serum have been reported that employ detection based on UV absorbance at 200 nm [9] or fluorescence following complex post-column derivatization [10]. However, HPLC serum assays employing electrochemical detection (ED) provided simpler, more specific procedures with equal or better sensitivity [11–16]. HPLC–ED serum assays have also been described for the macrolide antibiotics roxithromycin [16,17] and dirithromycin [18].

Most of these HPLC–ED methods employed reversed-phase chromatography using bonded-phase silica columns at pH of approximately 7 and coulometric detection by the ESA 5100A Coulochem detector. Amperometric detection of erythromycin by a glassy carbon electrode was also sensitive, but response rapidly decreased upon repeated injections of erythromycin due to electrode passivation [12–14]. Nevertheless, an HPLC serum assay using amperometric detection was reported [13]; detector sensitivity decreased at a rate of 60% in 24 h, requiring

daily cleaning of the electrode with acid and subsequent detector equilibration. A recent HPLC–ED plasma assay for erythromycin employed amperometric detection and a PRP-1 (non-ionic polystyrene–divinylbenzene copolymeric stationary phase) column eluted with an alkaline mobile phase [14]. Electrochemical detector response was more stable, although improved column chromatographic efficiency and consistency was desirable.

Chromatography conditions for HPLC–UV analysis of azithromycin in bulk product and in acid stability studies have been described [3,19], but HPLC assays in serum or tissue have not been described. This paper describes two HPLC–ED assays for azithromycin in serum and a variety of tissues. One assay, employed only for serum, uses a bonded-phase silica column and coulometric detection and is a modification of a method previously developed for erythromycin by Duthu [12]. A second assay, useful for both serum and tissue, employs a bonded-phase alumina column that enabled reversed-phase chromatography under alkaline conditions (pH of 10-11) and amperometric detection. Descriptions of these assays were presented in part in ref. 20.

EXPERIMENTAL

Materials

Azithromycin (I), internal standards II and III (the 9a-N-*n*-propyl analogue and 9a-N-propargyl analogue of azithromycin, respectively), and potential azithromycin metabolites 9a-N-desmethylazithromycin (IV), 3′-N-desmethylazithromycin (V), descladinoseazithromycin (VI), azithromycin-3′-N-oxide, and azithromycin-9a,3′-di-N-oxide (Fig. 1) were prepared by Pfizer (Groton, CT, U.S.A.)[a]. Erythromycin A was obtained from Upjohn Labs. (Kalamazoo, MI, U.S.A.). Hexane, acetonitrile, methanol, methyl-*tert.*-butyl ether, and water were all HPLC grade and purchased from Burdick and Jackson (Muskegon, MI, U.S.A.). All other chemicals were reagent grade.

Cyclic voltammetry

Cyclic voltammetry of azithromycin and erythromycin was conducted on a BAS CV-1B cyclic voltammograph (Bioanalytical Systems, West Lafayette, IN, U.S.A.) equipped with a glassy carbon working electrode and Ag/AgCl reference electrode. Compounds were dissolved in mobile phase to give a final concentration of 0.5 mg/ml. Current was recorded over a voltage range of -0.4 to $+1.3$ V at a scan rate of 5 mV/s.

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[a] Compounds prepared by Pfizer are identified as follows: azithromycin (CP-62,993), internal standards II (CP-65,205) and III (CP-67,094), 9a-N-desmethylazithromycin (CP-60,273), 3′-N-desmethylazithromycin (CP-64,434), descladinoseazithromycin (CP-66,458), azithromycin-3′-N-oxide (CP-63,358), and azithromycin-9a,3′-di-N-oxide (CP-63,029).

Serum assay using coulometric detection (coulometric assay)

HPLC instrumentation. A BAS PM-48 pump (Bioanalytical Systems) was equipped with a glass bead guard column (21 mm × 3.0 mm I.D., 40 μm particle size, Waters Assoc., Milford, MA, U.S.A.) and a reversed-phase Chromegabond alkylphenyl analytical column (50 mm × 4.6 mm I.D., 5 μm particle size, ES Industries, Marlton, NJ, U.S.A.). Analytes were detected with an ESA 5100A Coulochem electrochemical detector (Environmental Sciences Assoc., Bedford, MA, U.S.A.), which consisted of an ESA 5020 guard cell and an ESA 5010 dual-electrode analytical cell. The guard cell (+1.0 V) was placed in line before the injector to electrolyze components of the mobile phase and reduce background current. The column effluent was monitored with the dual-electrode detector operated in the oxidative screen mode. The applied potentials of the screen and detector electrodes were +0.7 and +0.8 V, respectively. Stainless-steel 0.5-μm filters (Rainin, Woburn, MA, U.S.A.) were placed in line before the guard cell and analytical cells to protect their porous carbon electrodes. Samples were automatically injected with an ISS-100 autosampler (Perkin-Elmer, Norwalk, CT, U.S.A.). Detector output was acquired by an SP-4200 computing integrator (Spectra-Physics, San Jose, CA, U.S.A.) operated in the peak-height mode at an attenuation of 8 mV.

HPLC mobile phase preparation. The mobile phase was a mixture of 0.02 *M* ammonium acetate–0.02 *M* sodium perchlorate–acetonitrile–methanol (22:23:45:10, v/v). The apparent pH of the mixture was adjusted to between 6.8 and 7.2 with concentrated acetic acid. Depending on the performance of a given column, however, the optimal composition and pH of the mobile phase may have to be slightly modified. The mixture was filtered through a 0.2-μm Nylon 66 filter (Rainin). The flow-rate was 1.0 ml/min. The mobile phase was recycled into a 1-l reservoir and replaced weekly.

Serum sample preparation. Azithromycin serum concentrations were determined using standards prepared in two concentration ranges, a low range of 0.020–0.25 μg/ml and a high range of 0.20–2.0 μg/ml. Aliquots (0.5 ml) of serum were pipetted into 16 × 125 mm disposable glass culture tubes. To each sample was added the internal standard II in 25 μl of acetonitrile in amounts of 0.25 μg for the low concentration range and 2.5 μg for the high concentration range. For an assay range of 0.010–0.10 μg/ml, 1.0-ml serum aliquots and 0.25 μg of internal standard II were utilized. Serum samples were alkalinized with 1.0 ml of 0.03 *M* potassium carbonate and mixed on a vortex mixer. The diluted samples were extracted with 5 ml of methyl-*tert.*-butyl ether for 30 s on a vortex mixer and centrifuged at 2000 *g* for 10 min. The ether layer was transferred to clean glass culture tubes and evaporated to dryness at 37°C under reduced pressure in a vortex evaporator (Buchler Instruments, Fort Lee, NJ, U.S.A.). The residue was reconstituted in 0.3 ml of mobile phase for 30 s with a vortex mixer and then washed with 0.3 ml hexane on a vortex mixer. The phases were separated by centrifugation and the hexane layer discarded. The aqueous layer was transferred

to injection vials, and 100-μl aliquots were injected onto the HPLC system with detector gain settings of 10 × 99 or 10 × 20, respectively, for the low and high concentration ranges.

Serum and tissue assay using amperometric detection (amperometric assay)

HPLC instumentation. A Spectroflow 400 pump (Kratos, Ramsey, NJ, U.S.A.) was equipped with a glass bead guard column (21 mm × 3.0 mm I.D., particle size 40 μm, Waters Assoc.) and a reversed-phase Chromegabond γRP-1 alumina analytical column (150 mm × 4.6 mm I.D., 5 μm particle size, ES Industries). An SSI LP-21 low pulse damper (Rainin) was placed in line prior to the injector to suppress pump pulsations. Column effluent was monitored with a BAS LC-4B amperometric electrochemical detector (Bioanalytical Systems), equipped with a glassy carbon electrode set at an oxidation potential of +0.8 V relative to an Ag/AgCl reference electrode. Samples were automatically injected with an ISS-100 autosampler (Perkin-Elmer). Detector output was acquired by an SP-4200 computing integrator (Spectra-Physics) operated in the peak-height mode at an attenuation of 8 mV.

HPLC mobile phase preparation. The mobile phase was a mixture of 0.05 *M* potassium phosphate–acetonitrile (70:30, v/v). The mixture was adjusted to apparent pH 11.0 with 1.0 *M* potassium hydroxide and filtered through a 0.2-μm Nylon 66 filter (Rainin). The flow-rate was 1.0 ml/min. The mobile phase was recycled into a 1-l reservoir and replaced weekly.

Serum sample preparation. Azithromycin serum concentrations were determined using standards prepared in two concentration ranges, a low range of 0.010–0.20 μg/ml and a high range of 0.20–2.0 μg/ml. Aliquots (0.5 ml) of serum were pipetted into 16 × 125 mm disposable glass culture tubes. Internal standard III was added in 25 μl of acetonitrile to each sample in amounts of 0.05 μg for the low concentration range and 0.5 μg for the high concentration range. Serum samples were alkalinized with 0.5 ml of 0.06 *M* potassium carbonate and mixed on a vortex mixer. The diluted samples were extracted with 5 ml of methyl-*tert.*-butyl ether for 30 s on a vortex mixer and centrifuged at 2000 *g* for 10 min. The ether layer was transferred to clean glass culture tubes. For serum samples in the low concentration range only, the ether layer was extracted with 0.5 ml of 0.015 *M* citric acid (pH 3.1) on a vortex mixer for 30 s and centrifuged at 2000 *g* for 5 min. Following removal of the ether layer by aspiration, the aqueous layer was alkalinized with 1.0 ml of 0.06 *M* potassium carbonate and mixed for 1 min on a vortex mixer. The aqueous layer was then extracted with 5 ml of methyl-*tert.*-butyl ether for 30 s on a vortex mixer and centrifuged at 2000 *g* for 10 min, and the ether layer was transferred to clean glass culture tubes. This procedure of back-extraction from ether into citric acid solution followed by re-extraction into ether after basification was not utilized for serum samples in the high concentration range. Then, for all serum samples, the final ether layer was evaporated to dryness at 37°C under reduced pressure in a vortex evaporator (Buchler Instru-

ments). The residue was reconstituted in 0.3 ml (low concentration range samples) or 0.5 ml (high concentration range samples) of a mixture of acetonitrile–water (1:1, v/v), mixed for 30 s on a vortex mixer, and then washed with one volume of hexane on a vortex mixer. The phases were separated by centrifugation, and the hexane layer was discarded. The aqueous layer was transferred to injection vials, and 60-μl aliquots were injected onto the HPLC system with the detector range settings at 5 and 10 nA, respectively, for the low and high concentration ranges.

Tissue sample preparation. Azithromycin tissue concentrations were determined using standards prepared in four concentration ranges between 0.1 and 1000 μg/g. An accurately weighed portion (approximately 1.0 g) of each tissue sample was placed in 100-ml centrifuge tubes. Nine volumes of acetonitrile were added to each tube. Internal standard III was added in 50–200 μl of methanol in amounts of 1.0, 5.0, 50, and 500 μg for the following concentration ranges, respectively: 0.1–2, 1–10, 10–100, and 100–1000 μg/g. The sample was homogenized with a Polytron PT 10/35 tissue homogenizer (Brinkmann Instruments, Westbury, NY, U.S.A.) for 10 s and then centrifuged at 2000 *g* for 10 min. An 0.5-ml aliquot of each supernatant was pipetted into 16 × 125 mm disposable glass culture tubes and evaporated to dryness at 50°C under reduced pressure in a vortex evaporator (Buchler Instruments). The residue was reconstituted in 0.5 ml of 0.06 *M* potassium carbonate and extracted with 5 ml of methyl-*tert.*-butyl ether for 30 s on a vortex mixer and centrifuged at 2000 *g* for 10 min. The ether layer was transferred to clean glass culture tubes and evaporated to dryness at 37°C under reduced pressure in a vortex evaporator. The residue was reconstituted in 1.0 ml (0.3 ml for the 0.1–2 μg/g concentration range) of a mixture of acetonitrile–water (1:1, v/v), mixed for 30 s on a vortex mixer, and then washed with one volume of hexane. Following separation of the phases by centrifugation and removal of the hexane layer, the aqueous layer was transferred to polyethylene injection vials and a 20–60 μl aliquot was injected onto the HPLC system with detector range settings of 5–20 nA as appropriate for the concentration range.

Assay calibration

Assay calibration standards were prepared at a single concentration appropriate for the range of azithromycin concentrations anticipated in the test serum and tissue samples. Blank serum samples were fortified with azithromycin in quadruplicate at concentrations of 0.05 or 0.1 μg/ml for the low concentration range and 0.5 μg/ml for the high concentration range. Blank tissue samples were fortified prior to homogenization with azithromycin in quadruplicate at concentrations of 0.6 or 1.0, 4.0, 50, and 500 μg/g for the four concentration ranges described above in the preparation of tissue samples for assay. The mean response factor (product of the standard azithromycin concentration times the peak-height ratios of internal standard/azithromycin standard) of the calibration standards was deter-

mined. Azithromycin concentrations of unknown serum or tissue homogenate samples (μg/ml) were calculated from the product of the mean response factor times the peak-height ratios of azithromycin/internal standard in the test sample. The concentration of azithromycin per gram of tissue was then calculated from the product of the tissue homogenate concentration times the ratio of total tissue homogenate volume/sample weight. To verify assay linearity and that standard curves had y-intercepts similar to zero, four- or five-point standard curves were prepared by fortifying control serum or tissue with azithromycin over all concentration ranges of interest.

RESULTS

Electrochemical detection

Cyclic voltammetry of azithromycin at pH 7 in the mobile phase used for the coulometric serum assay showed an anodic wave with $E_{1/2}$ of +0.80 V (Fig. 2). A lower $E_{1/2}$ of +0.64 V was observed at pH 11 in the alkaline mobile phase used for the amperometric serum and tissue assay. Peak currents were similar at both pH values. Oxidation potentials of erythromycin were similar to those of azithromycin at both pH values, while peak currents of erythromycin were about half those of azithromycin.

Hydrodynamic voltammograms of the oxidation of azithromycin under HPLC conditions also showed that maximum peak-height response occurred at approximately +0.95 V in pH 6.8 mobile phase in the coulometric assay and at +0.80 V in pH 11 mobile phase in the amperometric assay. Peak height responses for 9a-N-desmethylazithromycin and 3′-N-desmethylazithromycin were each approximately half that of azithromycin when equimolar amounts were injected under HPLC conditions of both assays. In the amperometric assay, peak height response for azithromycin-3′-N-oxide was also approximately half that of azithromycin; no electrochemical response was observed for azithromycin-9a,3′-di-N-oxide.

Chromatography

Chromatograms of azithromycin and internal standard II for the coulometric assay in human serum are shown in Fig. 3. HPLC profiles of azithromycin and internal standard III for the amperometric assay in human serum and tissues are shown in Figs. 4 and 5. Similar chromatograms were obtained with serum and tissues from rats, dogs, mice, and rabbits. Azithromycin and internal standard eluted at about 9 and 13 min in the coulometric assay and at about 8 and 10 min in the amperometric assay. Interfering peaks eluting in the regions of azithromycin and internal standard were not found in blank serum or tissues. Occasionally, in the coulometric serum assay, slight adjustment of mobile phase pH between 6.8 and 7.2 was necessary to separate analyte peaks from small background peaks. Possible azithromycin metabolites — 3′-N-desmethylazithromycin, 9a-N-des-

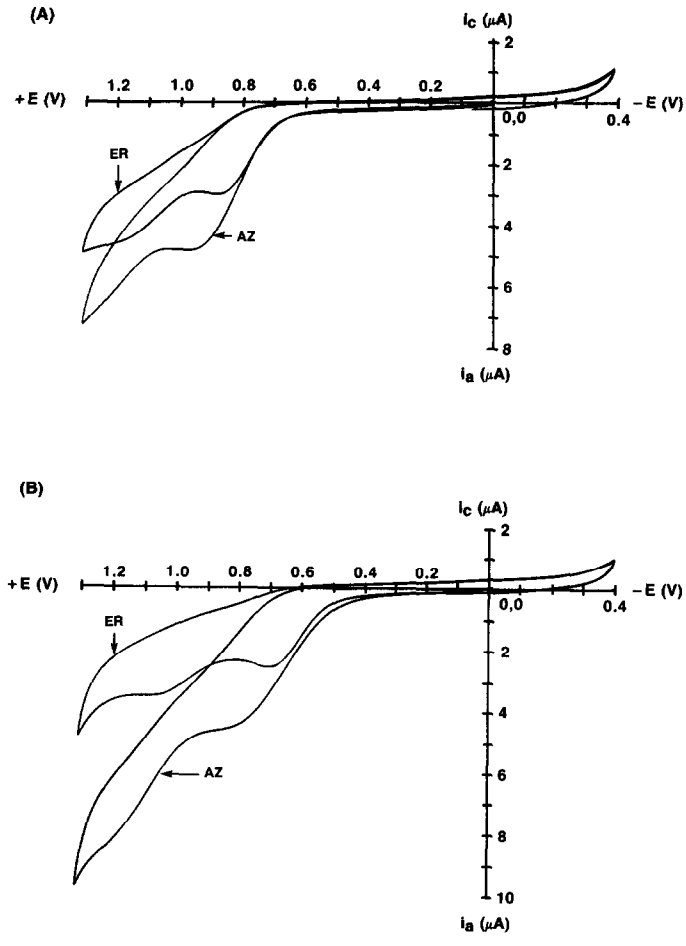

Fig. 2. Cyclic voltammograms of azithromycin (AZ) and erythromycin (ER). (A) AZ and ER (0.5 mg/ml) in the mobile phase used for the coulometric serum assay (0.02 *M* ammonium acetate–0.02 *M* sodium perchlorate–acetonitrile–methanol, 22:23:45:10, v/v, pH 7.0). (B) AZ and ER (0.5 mg/ml) in the mobile phase used for the amperometric serum and tissue assay (0.05 *M* potassium phosphate-acetonitrile, 70:30, v/v, pH 11.0).

methylazithromycin, descladinoseazithromycin, and azithromycin-3′-N-oxide — eluted earlier than azithromycin and did not interfere in the determination of azithromycin (Table I).

Linearity

For the coulometric and amperometric assays, standard curves constructed with four or five different concentrations of azithromycin in blank serum and tissues were linear over the indicated concentration ranges, with correlation coefficients greater than 0.997 and *y*-intercepts of nearly zero (Table II). Standard

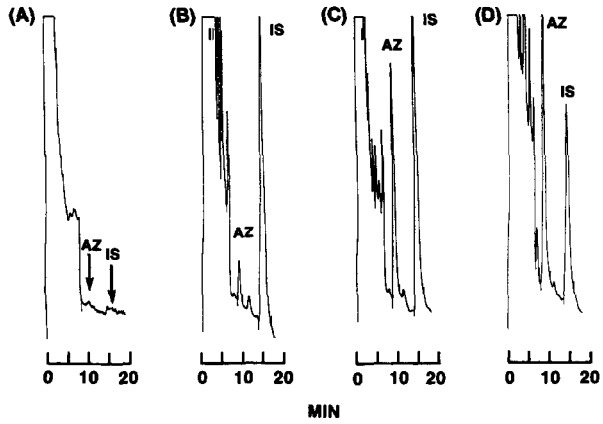

Fig. 3. HPLC–ED of extracted serum samples for coulometric serum assay. Peaks: AZ = azithromycin; IS = internal standard. (A) Control human serum (gain 10 × 99); arrows indicate elution times of azithromycin and internal standard. (B) Control human serum fortified with 0.02 μg/ml azithromycin (gain 10 × 99). (C) Control human serum fortified with 0.5 μg/ml azithromycin (gain 10 × 20). (D) Serum collected from a subject 3 h following a single 500-mg oral dose of azithromycin (gain 10 × 99); azithromcyin concentration 0.15 μg/ml.

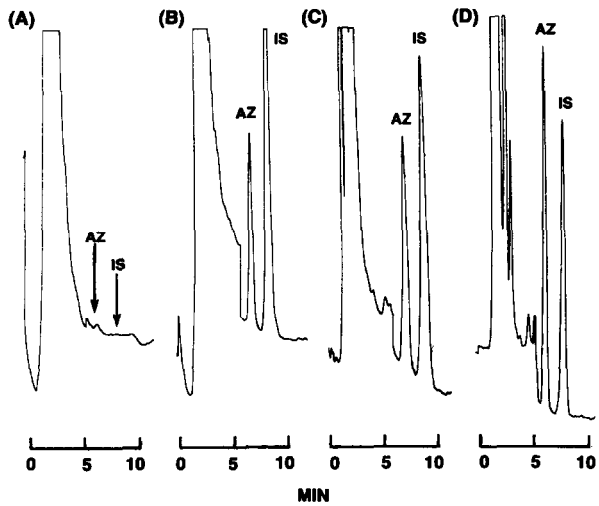

Fig. 4. HPLC–ED of extracted serum samples for amperometric serum assay. Peaks: AZ = azithromycin; IS = internal standard. (A) Control human serum (range 5 nA); arrows indicate elution times of azithromycin and internal standard. (B) Control human serum fortified with 0.05 μg/ml azithromycin (range 5 nA). (C) Control human serum fortified with 1.0 μg/ml azithromycin (range 10 nA). (D) Serum collected from a subject 2 h following a single 500-mg oral dose of azithromycin (range 5 nA); azithromycin concentration 0.14 μg/ml.

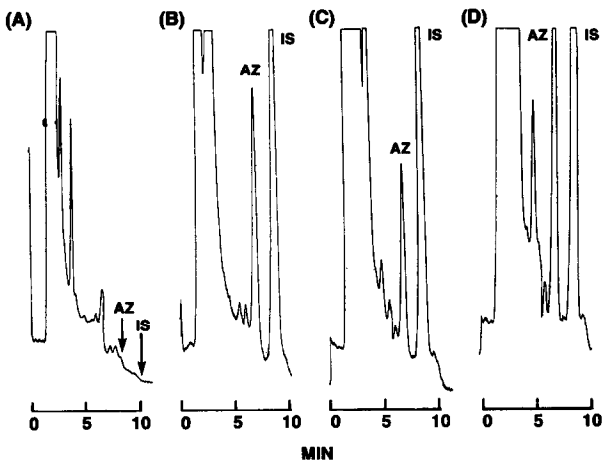

Fig. 5. HPLC–ED of extracted tissue samples for amperometric tissue assay. Peaks: AZ = azithromycin; IS = internal standard. (A) Control human uterus (range 5 nA); arrows indicate elution times of azithromycin and internal standard. (B) Control human uterus fortified with 2.0 μg/mg azithromycin (range 5 nA). (C) Human uterus collected from a subject 14 h following a single 500-mg oral dose of azithromycin (range 5 nA); azithromycin concentration 1.4 μg/g. (D) Human tonsil collected from a subject 24 h following a single 500-mg oral dose of azithromycin (range 5 nA); azithromycin concentration 3.9 μg/g.

curves in serum and tissues constructed over a greater concentration range than those indicated seemed to deviate slightly from linearity. Therefore, use of separate standard curves prepared over smaller concentration ranges assured assay linearity within each working range. As expected, the reciprocals of mean re-

TABLE I

HPLC RETENTION OF AZITHROMYCIN AND POTENTIAL METABOLITES OF AZITHROMYCIN

Compound	Capacity factor k'	
	Coulometric assay[a]	Amperometric assay[b]
Erythromycin A	–	1.7
Azithromycin-3′-N-oxide	–	1.5
Descladinoseazithromycin	2.6	2.2
3′-N-Desmethylazithromycin	6.2	3.1
9a-N-Desmethylazithromycin	6.4	4.3
Azithromycin	7.6	5.3

[a] Column: Chromegabond alkylphenyl, 5 μm, 50 mm × 4.6 mm I.D.; mobile phase: 0.02 *M* ammonium acetate–0.02 *M* sodium perchlorate–acetonitrile–methanol (22:23:45:10, v/v), pH 6.8; flow-rate: 1.0 ml/min.

[b] Column: Chromegabond γRP-1 alumina, 5 μm, 150 mm × 4.6 mm I.D.; mobile phase: 0.05 *M* potassium phosphate–acetonitrile (70:30, v/v), pH 11; flow-rate: 1.0 ml/min.

TABLE II

CALIBRATION DATA FOR COULOMETRIC SERUM AND AMPEROMETRIC SERUM AND TISSUE ASSAYS OF AZITHROMYCIN

Sample	Concentration range (μg/ml or μg/g)	Slope	Intercept	Correlation coefficient	Calibration concentration for response factor (μg/ml or μg/g)	Reciprocal of response factor
Coulometric assay						
Serum	0.010–0.10	19.769	0.035	0.9991	0.050	21.34
	0.020–0.25	5.591	−0.004	0.9998	0.10	5.68
	0.20–2.0	0.719	−0.038	0.9998	0.50	0.684
Amperometric assay						
Serum	0.010–0.20	11.431	0.035	0.9973	0.050	12.16
	0.20–2.0	1.558	−0.032	0.9988	0.50	1.42
Brain	0.10–2.0	1.299	0.065	0.9971	1.0	1.433
Muscle	0.20–2.0	1.390	0.018	0.9993	0.60	1.389
Liver	1.0–10	0.288	−0.061	0.9997	4.0	0.264
Kidney	10–100	0.0198	−0.069	0.9996	50	0.0175
Kidney	100–1000	0.00209	0.0007	0.9987	500	0.00217

sponse factors were similar to the slopes determined from the multi-level standard curves. Therefore, assay calibration by determination of response factors at a single concentration was an acceptable method for the analysis of unknown samples.

Precision and accuracy

Assay accuracy and within-run precision results, determined from replicate serum and tissue samples over all concentration ranges, are summarized in Table III. For the coulometric and amperometric assays, intra-assay precision ranged from 1 to 8% (relative standard deviation) for serum and tissues. Accuracy of measured concentrations was within 1–11% of fortified concentrations in serum or tissues. Inter-assay precision of replicate serum or tissue samples (n = 4–12) prepared over all concentration ranges ranged from 4 to 11% (relative standard deviation) during a two-month analysis period. Based on an intra-assay precision of less than 20% and accuracy within 20%, the lower limit of quantification of azithromycin in serum was 0.01 μg/ml for the coulometric and amperometric assays; the lower limit in tissues was 0.1 μg/g.

Recovery

Assay recovery of azithromycin was determined by comparing peak heights of azithromycin isolated from fortified serum or tissues to that of standards injected

TABLE III

ACCURACY AND PRECISION OF INTRA-ASSAY DETERMINATIONS OF BLANK SERUM AND TISSUE FORTIFIED WITH AZITHROMYCIN

Sample	Added concentration (μg/ml or μg/g)	*n*	Intra-assay	
			Measured concentration (μg/ml or μg/g)	Precision R.S.D. (%)
Coulometric assay				
Serum	0.010	5	0.010	4.3
	0.020	4	0.019	4.3
	0.10	4	0.100	2.3
	0.50	5	0.444	1.8
	2.0	5	2.04	1.0
Amperometric assay				
Serum	0.010	2	0.0099	–
	0.020	3	0.018	7.8
	0.10	3	0.106	6.0
	0.5	4	0.488	2.0
	2.0	4	2.18	2.3
Brain	0.10	2	0.093	–
Muscle	0.20	3	0.22	3.3
Liver	2.0	4	1.88	2.7
Liver	10	4	10.5	5.5
Kidney	50	4	50.5	1.1
Kidney	200	4	198	4.8
Kidney	1000	4	950	4.6

directly onto the HPLC column. Mean recoveries of azithromycin from rat serum (0.1 μg/ml) and human serum (0.5 μg/ml) were 88 and 86%, respectively. Those from rat liver (10 μg/g) and kidney (50 μg/g) were 85 and 84%, respectively.

Stability

Azithromycin in fortified serum or in human tissues from dosed patients was stable for at least two and three months, respectively, when stored frozen at −20°C. No degradation of azithromycin or internal standard was observed while storing extracted serum or tissues samples reconstituted in a mixture of acetonitrile–water (1:1, v/v) or in the mobile phases of the coulometric or amperometric HPLC systems at room temperature for 24 h, indicating that azithromycin is stable under HPLC conditions.

DISCUSSION

Chromatography

Chromatography of azithromycin on bonded-phase silica columns at pH 6.8-7.2 is hampered by peak tailing, perhaps due to interaction of the protonated amine functions of azithromycin with the silica. Short columns were necessary, as excessive length resulted in peak broadening or failure of azithromycin to elute. Amongst several stationary phases tested at pH 7 in the coulometric serum assay, the alkylphenyl column (ES Industries) yielded minimal peak tailing and optimal separation from background peaks. A Novapak C_{18} column (Waters Assoc.) and a diphenyl column (Analytichem International, Harbor City, CA, U.S.A.) have also been employed. The ionic strength of the mobile phase (20 m*M* ammonium acetate–20 m*M* sodium perchlorate–acetonitrile–methanol, 22:23:45:10, v/v) was sufficiently high to minimize peak tailing but low enough to minimize background current. Sodium perchlorate in the mobile phase prolonged electrode sensitivity [12]. The pH of the mobile phase greatly affected the retention of azithromycin, with shorter retention times as the pH was lowered. However, electrochemical oxidation was facilitated at higher pH. The optimal pH of the mobile phase was between 6.8 and 7.2.

While the alkylphenyl column was successfully used in many studies, a lack of reproducibility in retention time or peak shape among columns was occasionally noted. This was corrected by appropriate changes in the mobile phase pH or composition or by column replacement. Column life, as evidenced by maintenance of peak shape, was also variable, lasting from several weeks to months. Recently, a Zorbax RX reversed-phase C_8 bonded silica column (introduced by DuPont with improved silica technology for the analysis of basic compounds) was found to produce good peak shape for azithromycin and may prove useful in the coulometric serum assay.

Chromatography of azithromycin was improved by utilization of a γRP-1 reversed-phase alumina column with an alkaline mobile phase. The γRP-1 column contains alumina particles coated with polymers cross-linked by γ-irradiation and is similar to organic-polymer columns with respect to surface interactions and pH stability. Chromatography under alkaline conditions (pH of 10–11), in which azithromycin is deprotonated (pK_a 8.1 and 8.8), resulted in improved peak shape. The ionic strength of the mobile phase (50 m*M* potassium phosphate–acetonitrile, 70:30, v/v) was optimal for good peak shape and minimal background current. Supplementation of the mobile phase with sodium perchlorate was not necessary for maintenance of electrode sensitivity. Azithromycin retention times varied between pH 10 and 11, but pH 11 offered optimal separation from background peaks in serum and tissue extracts. Chromatography on the γRP-1 columns was reproducible from column to column. The γRP-1 columns had long lifetimes, with no significant decrease in column performance over a period of six months or more involving several thousand injections of

extracts of biological samples. Attempts to chromatograph azithromycin on a PRP-1 column with an alkaline mobile phase (Hamilton, Reno, NV, U.S.A.), recently employed in an erythromycin plasma assay [14], produced unsatisfactory peak shapes, confirming the results of Kovacic-Bosnjak *et al.* [19].

Electrochemical detection

Cyclic voltammetric studies and HPLC–ED responses with azithromycin and N-desmethyl and N-oxide analogues of azithromycin indicated that oxidation of both tertiary amine groups in azithromycin (3′ and 9a positions) is responsible for the electroactivity of azithromycin. The approximately two-fold greater peak current of azithromycin compared with that of erythromycin was consistent with the presence of a second electroactive tertiary amine. Both mono-N-demethylated analogues of azithromycin (3′-N-desmethylazithromycin and 9a-N-desmethylazithromycin) and azithromycin-3′-N-oxide had HPLC–ED peak-height responses approximately half that of azithromycin. The lack of an HPLC–ED response with azithromycin-9a,3′-di-N-oxide further suggested the dependence of electroactivity on both tertiary amine functions. These results are consistent with those reported for erythromycin, whose N-desmethyl and N,N-didesmethyl analogues lack electrochemical activity [12].

Electrochemical oxidation of azithromycin in its deprotonated form was facilitated compared with its oxidation in its protonated form, as evidenced by the lower $E_{1/2}$ at pH 11 compared with pH 7. The sensitivity of azithromycin to ED, however, was the same at both pH values, as the similar peak currents at pH 7 and 11 indicated that the rate of oxidation was diffusion-limited at both pH values.

For the coulometric serum assay, detection was accomplished with the dual electrodes operated in the oxidative screen mode, as suggested by several investigators reporting similar HPLC–ED assays for erythromycin and roxithromycin [11,12,16,17]. Little oxidation of azithromycin occurred at the first electrode set at a screen potential of +0.7 V. Although greater response could be obtained for azithromycin with higher potentials than +0.8 V at the second electrode, the background noise also increased. Therefore, the potential of +0.80 V was selected to provide adequate sensitivity for azithromycin determination in serum and minimal background. Routine operation at a detector gain setting of 10 × 99 was possible, and the limit of detection for azithromycin injected directly onto the column was approximately 1 ng. Electrode response slowly decreased upon repeated injections of serum extracts over a period of several weeks, but response could be restored by acid washing the detector cell with 6 *M* nitric acid for 30 min (rinsing the cell before and after with acetonitrile–water, 1:1, v/v) followed by overnight washing with 20 m*M* sodium perchlorate–acetonitrile (1:1, v/v).

In the initial development of an assay using the γRP-1 alumina column with its alkaline mobile phase, the detection limit with the ESA coulometric detector was

restricted by a noisier baseline due to higher background currents. In addition, high pressure build-up occurred due to plugging of the 0.5-μm filter placed in line before the guard cell. Use of the BAS glassy carbon amperometric electrochemical detector eliminated these problems. This detector could be operated at +0.80 V, the potential at which peak current was produced, with little baseline noise (background current was typically 80–120 nA). Routine operation at detector range settings as low as 5 nA full scale was possible, permitting a detection limit of about 1 ng of azithromycin injected on-column.

When the BAS glassy carbon amperometric detector was used with the alkylphenyl bonded-phase silica column eluted by mobile phases at approximately pH 7, repeated injections of azithromycin caused rapid loss of sensitivity due to electrode passivation. This was analogous to reports of passivation of glassy carbon electrodes with repeated injections of erythromycin in similar HPLC systems [12–14]. Consequently, the use of the ESA coulometric detector was required in this HPLC system. In contrast, rapid electrode passivation did not occur when the BAS amperometric detector was used with the γRP-1 alumina column with its alkaline mobile phase. Loss in detector sensitivity was very gradual, and repeated injections (1000 or more) of azithromycin tissue and serum extracts could be made over a period of several months before re-polishing of the electrode surface was necessary.

Applications

The coulometric serum and the amperometric serum and tissue assays for azithromycin have been used successfully in our laboratory for the last three years to analyze thousands of samples. Azithromycin has been determined in serum and a large variety of tissues (including liver, kidney, lung, spleen, muscle, fat, brain, tonsil, lymph nodes, eye, prostate and other urological tissues, and gynecological tissues) from rats, dogs, mice, and rabbits, as well as humans [5,6]. The amperometric assay has also been applied to other biological fluids, including urine, bile, cerebrospinal fluid, prostatic fluid, and peritoneal fluid. Approximately forty serum or twenty tissue samples per day can be assayed by a single operator; the limiting factor is the time required for sample preparation and not the HPLC analysis. The HPLC system with the ESA coulometric detector has only been employed for the assay of serum samples, but may possibly be useful for tissue samples. The HPLC assay utilizing the γRP-1 alumina column with its alkaline mobile phase has been favored over that using the alkylphenyl silica column because of improved, more consistent chromatography and longer column life.

The limited dynamic range of the assays required calibration over several concentration ranges to assure linearity of assay responses. For determination of serum and tissue azithromycin concentrations in humans following 250- and 500-mg doses, the calibration ranges of 0.01–0.2 μg/ml in serum and 0.1–2 and 1–10 μg/g in tissues were most useful [6]. Samples containing azithromycin levels outside these ranges were re-assayed with an appropriate calibration curve.

Slopes of the amperometric assay calibration curves prepared over several concentration ranges in serum, plasma, liver, kidney, muscle, and fat from humans, rats, and dogs were compared, following adjustment for their respective internal standard concentrations to yield a normalized response factor. The low variability (relative standard deviation of 5.4%) among the normalized response factors indicated that calibration curves and the isolation of azithromycin and internal standard were not affected by tissue type and species origin.

CONCLUSIONS

Azithromycin can be determined in serum and a large variety of tissues with good accuracy and precision using HPLC–ED. Analysis can be accomplished by reversed-phase chromatography using either a bonded-phase silica column with approximately neutral mobile phase and detection by an ESA coulometric detector or by a γRP-1 alumina column with an alkaline mobile phase and detection by a BAS amperometric detector. The HPLC–ED assays have low limits of quantification and are able to measure azithromycin over a wide range of concentrations. No metabolites of azithromycin or endogenous substances found in serum and tissues are known to interfere with the determination of azithromycin. The HPLC analysis is automated, which is convenient and allows high sample throughput. The assays could be used to quantify microbiologically active and inactive metabolites of azithromycin, as well as degradation products, that retain tertiary amine groups.

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