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Short Communication

Equivalence of a high-performance liquid chromatographic assay and a bioassay of azithromycin in human serum samples

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ABSTRACT

Two sensitive methods for the determination of the azalide antibiotic azithromycin in human serum were compared. High-performance liquid chromatography (HPLC) and a microbiological assay were simultaneously applied to 768 serum samples obtained in a clinical study. There was excellent agreement between the azithromycin concentrations measured by HPLC and by the bioassay. The correlation coefficient for the two methods was $r^2 = 0.96$. The precision and the sensitivity of the methods were found to be very similar.

INTRODUCTION

Azithromycin (9-deoxo-9a-methyl-9a-aza-9a-homoerythromycin A) (Pfizer, New York, NY, USA) is an antibiotic of a novel class known as azalides, which contain a nitrogen atom in the macrolide aglycone ring. The antibiotic is particularly suited for the treatment of upper and lower respiratory tract infections and for skin and soft tissue infections in both adults and children [1]. In comparison with erythromycin, the new antibiotic exhibits a broader spectrum of antimicrobial activity and more reliable oral absorption. Further, azithromycin shows an extended half-life of elimination and a favourable tissue distribution [2–4]. Two methods have been used to measure azithromycin in body fluids and tissues: a microbiological assay employing agar plate diffusion techniques [5–7] and high-performance liquid chromatography (HPLC) with electrochemical detection [8]. In view of the methodological differences between the two assays, it was considered of interest to examine whether pharmacokinetic data produced by the two methods are interchangeable.

EXPERIMENTAL

Materials

Azithromycin, the internal standard (*n*-propyl analogue of azithromycin) and the azithromycin metabolites 3′-N-desmethylazithromycin (I), 9a-N-desmethylazithromycin (II), descladinose-azithromycin (III) and azithromycin-3′-N-oxide

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(IV) were obtained from Pfizer (Groton, CT, USA). Acetonitrile, hexane, methanol, methyl *tert.*-butyl ether and water were of HPLC grade.

Azithromycin HPLC assay

HPLC instrumentation. The chromatographic system included a Constametric I HPLC pump (Latek, Heidelberg, Germany), a Gilson M 231 automatic injector with a 50-μl sample loop (Abimed, Langenfeld, Germany) and a 125 mm × 4.6 mm I.D. analytical column with a 20 mm × 4.6 mm I.D. guard column filled with Nucleosil C_{18} (5 μm particle size) (Grom, Herrenberg, Germany). Analytes were detected with an ESA 5100 A Coulochem electrochemical detector (Bischoff, Leonberg, Germany), which consisted of a guard cell (+1 V) and a dual-electrode analytical cell (+0.7 and +0.8 V). The detector response was stable over 500–5000 injections, depending on the quality of the cell.

The mobile phase was 0.04 *M* disodium hydrogenphosphate–0.005 *M* tetrabutylammonium perchlorate–acetonitrile (50:50:33, v/v/v), adjusted to pH 7.0 with 25% phosphoric acid. The mobile phase, which was renewed about every 1000 injections, was recycled into a 6-l reservoir at a flow-rate of 1 ml/min.

Sample preparation and calibration. Standard samples were prepared by spiking 0.5 ml of serum with 25 μl of azithromycin solutions in methanol–water (20:80, v/v) to give six calibration points between 0.008 and 0.8 μg/ml. For the calculation of run-to-run and within-run reproducibilities, spiked serum samples were prepared. Specificity against azithromycin metabolites was tested by use of spiked serum samples.

The extraction and sample preparation as described by Shepard *et al.* [8] were used. These steps were adapted to a fully automated procedure on an assembly line (Programmable ASA Modular System; Ismatec, Zürich, Switzerland).

Linear calibration graphs were calculated on the basis of peak-height ratios of azithromycin to internal standard. Linearity was examined using the *F*-test.

Azithromycin microbiological assay

Azithromycin was determined in serum using an agar cylinder diffusion technique with *Sarcina lutea.* Serum samples of 0.3 ml were applied to the bioassay plates without further pretreatment. Triplicates of six clinical and six standard samples (range 0.008–0.25 μg/ml serum) were distributed over the same plate. Serum samples with expected concentrations higher than 0.25 μg/ml were appropriately diluted with phosphate buffer. The plates were incubated at 28°C for 18 h. Calibration graphs were constructed by plotting the diameters of the inhibition zones against the logarithm of the azithromycin concentrations. The assay reproducibility was calculated using the same spiked samples as for the HPLC assay.

Separate calibration graphs were set up for azithromycin and for each metabolite. From these calibration graphs those metabolite concentrations which produced the same zone diameter as 1 μg/ml azithromycin were calculated. The concentration ratio of azithromycin to metabolite was then used as a measure of the relative susceptibility of the bioassay to the metabolite.

Study protocol

In an open, two-way crossover study, two single oral doses of 600 mg of azithromycin were compared. Twenty-four healthy volunteers were included in this pharmacokinetic bioequivalence study. Serum samples of 4 ml were obtained at each of sixteen sampling times. The study was approved by an independent Ethical Review Board.

RESULTS AND DISCUSSION

Specificity, sensitivity, linearity and reproducibility of the HPLC assay

Azithromycin and the internal standard had retention times of 5.8 and 7.8 min, respectively. The respective peaks did not interfere either with the peaks coming from the biological matrix (Fig. 1) or with those from the metabolites of azithromycin (see Table II) which were coextracted.

The calibration graphs were linear over the

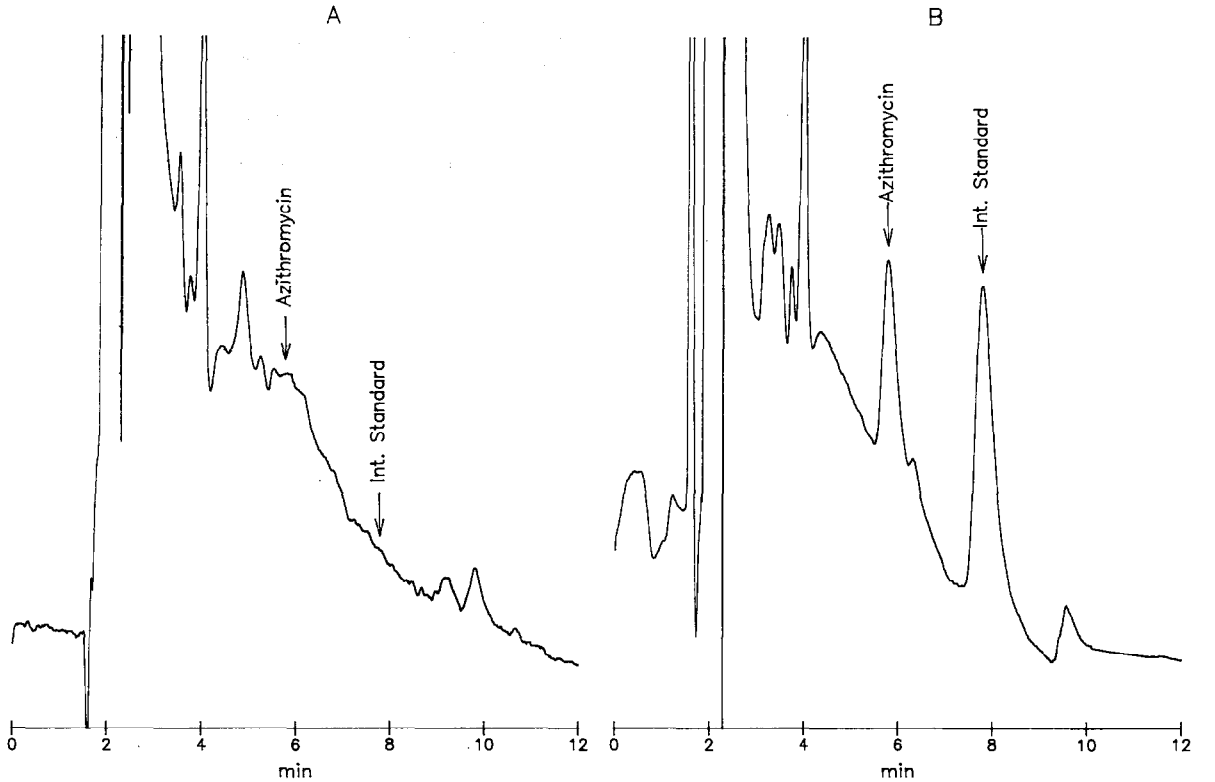

Fig. 1. HPLC of extracted serum samples. (A) Control human serum; (B) serum collected from a subject 2.5 h after a single 600-mg dose of azithromycin (measured concentration of azithromycin: 0.27 μg/ml).

tested range of concentrations ($p < 0.01$). The correlation coefficients with six calibration points were always greater than 0.997. We defined the lowest calibration point at 0.008 μg/ml as the lower limit of quantification of azithromycin. This concentration equals the measured mean concentration of twenty blank samples plus three times the standard deviation [9], at a signal-to-noise ratio of about 5:1.

The within-run and run-to-run reproducibili-

TABLE I

REPRODUCIBILITY OF THE HPLC ASSAY AND THE BIOASSAY

Method	Given concentration (μg/ml)	Run-to-run reproducibility		Within-run reproducibility	
		Found (μg/ml)	C.V. (%)[a]	Found (μg/ml)	C.V. (%)[a]
HPLC	0.025	0.024	5.9 (6)	0.021	5.6 (6)
	0.100	0.096	5.1 (6)	0.099	4.0 (6)
	0.400	0.397	4.4 (6)	0.413	1.7 (6)
Bioassay	0.025	0.024	9.4 (6)	0.025	6.0 (18)
	0.100	0.097	6.6 (6)	0.095	5.7 (18)
	0.400	0.380	4.0 (6)	0.390	5.0 (18)

[a] Coefficient of variation; number of replicates in parentheses.

TABLE II

SPECIFICITY OF THE HPLC ASSAY AND THE BIOASSAY FOR THE METABOLITES OF AZITHROMYCIN

Compound	HPLC assay		Bioassay
	Relative retention	Relative detector response (%) at 1 μg/ml	Susceptibility relative to 1 μg/ml azithromycin (%)
Azithromycin	1.00	100	100
Metabolite I	0.89	58	32
Metabolite II	0.63	52	87
Metabolite III	0.36	90	0
Metabolite IV	1.92	40	6

ties were in the range 1.7–5.9% at concentrations of 0.025, 0.1 and 0.4 μg/ml (Table I).

Specificity, sensitivity, linearity and reproducibility of the bioassay

The relationship between the logarithm of the azithromycin concentration and the diameter of the zone of inhibition was linear over the range of drug concentrations from 0.008 to 0.25 μg/ml. The correlation coefficients were consistently greater than 0.997 ($n = 6$ calibration points). The lowest detectable amount of azithromycin was 0.008 μg/ml.

Three of the metabolites of azithromycin also show zones of inhibition of *Sarcina lutea* on the agar plates. The analytical susceptibility of the assay to the metabolites ranged between 0 and 87% of the susceptibility to unchanged azithromycin (Table II).

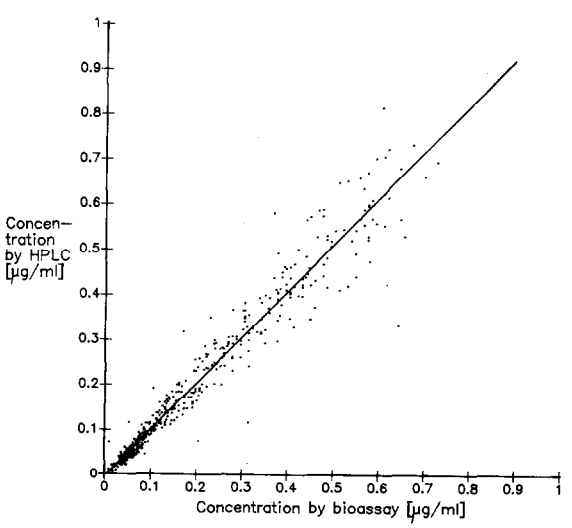

Fig. 2. Correlation of the serum concentrations of azithromycin obtained by HPLC and by bioassay.

Comparison of HPLC and bioassay

All serum samples obtained in the clinical study were analysed by both methods. The concentrations obtained with the two assays showed a linear correlation (Fig. 2). The equation of the fitted curve was $y = 1.03x - 0.002$ ($n = 668$; $r^2 = 0.96$). The serum level *versus* time curves obtained by the two methods showed good agreement, as exemplified by Fig. 3.

The two assays showed very similar within-run and run-to-run reproducibilities. With respect to specificity, it was demonstrated that the presence of metabolites does not interfere with the HPLC assay of azithromycin. The bioassay may cover metabolites in addition to azithromycin. However, on average the azithromycin concentrations measured by the bioassay were not higher than those obtained by HPLC. It is concluded that no significant amounts of metabolites were present in the serum samples analysed. The concentrations found in spiked samples were close to the given concentrations for both methods (Table I), suggesting good accuracy.

In conclusion, HPLC and bioassay of serum are similarly precise and sensitive. The two methods seem equally suitable for the determination of azithromycin concentrations in serum from patients after therapeutic doses of the drug.

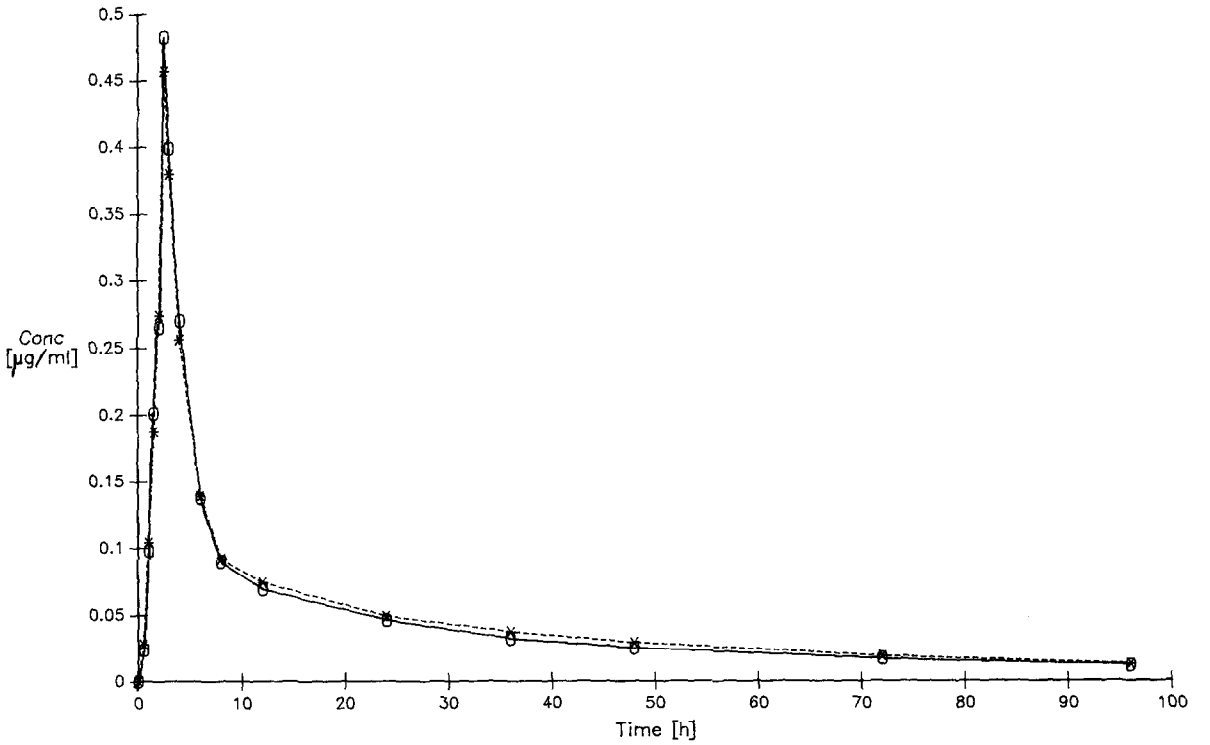

Fig. 3. Mean ($n = 24$) serum concentrations of azithromycin in healthy volunteers after a single dose of 600 mg of azithromycin. Drug determinations by HPLC (solid line) and bioassay (dashed line).

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