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Roxithromycin Bioassay Procedures for Human Plasma, Urine and Milk Specimens

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Roxithromycin (RU 28965 or RU 965) is a macrolide antibiotic with interesting pharmacokinetic properties. Its spectrum of antibacterial activity is similar to that of erythromycin (1–4), but blood levels are higher, more prolonged and more predictable (5, 6). Protein binding of roxithromycin has been found to be in excess of 95% (personal communication). Like erythromycin, roxithromycin is much more active in an alkaline medium. Both of those characteristics influence the selection of optimal conditions for a sensitive bioassay procedure. To date, chemical assay methods have not been reported, but bioassay procedures have been used. This report describes a standard bioassay procedure (7, 8) modified for measurement of roxithromycin in human plasma, urine, milk and mouse plasma, and used for the past two years.

Roxithromycin was kindly provided by Hoechst-Roussel Pharmaceuticals, USA. Stock solutions were prepared by dissolving the drug in a minimal volume of 95% ethanol and then diluting the solution with distilled water. The solutions were stored at -20°C or colder until needed. For assay of plasma samples, stock solutions of roxithromycin were diluted in normal human plasma or normal mouse plasma. At least seven doubling dilutions (ranging from 0.5 to 32 $\mu\text{g/ml}$) were normally tested. Control plasma samples containing 6.0 μg or 2.0 μg roxithromycin per ml were prepared and stored at -40°C . For assay of urine specimens, variability in the pH of different samples was minimized by diluting each specimen with equal volumes of 0.1 M Tris buffer (pH 7.9). The specimens were thoroughly mixed before a sample was removed for testing. Significantly lower assay values were obtained if the sediments were first

allowed to settle. The standard solutions were prepared in 0.1 M Tris buffer rather than using less predictable samples of urine from untreated subjects as diluents. Control samples were prepared in normal urine and stored at -40°C ; they were diluted with equal volumes of 0.1 M Tris just before testing. For assay of human milk, standard solutions were prepared in a predose sample from the subject being studied. Each set of standards included doubling dilutions of roxithromycin, ranging from 0.25 to 32 $\mu\text{g/ml}$. Controls containing 6.0 or 2.0 $\mu\text{g/ml}$ were prepared in milk from one subject. Predose specimens from each subject were also tested to detect non-specific inhibitory activity.

Large bioassay plates (33 cm $\times$ 33 cm) were prepared by attaching aluminium sides to sheets of plate glass using a nontoxic silicone glue. Antibiotic Medium No. 11 (Difco Laboratories, USA) was used for all assays. This medium has a pH of 8.0 after autoclaving: roxithromycin zones were much smaller in less alkaline media. The assay strain used was *Sarcina lutea* (ATCC 9341), colonies of which were selected

Figure 1: Standard curves for roxithromycin diluted in water, mouse plasma, human plasma and human milk. Each zone diameter represents the mean of triplicate zones, all on the same assay plate.

from an overnight blood agar plate and suspended in sterile saline. The suspension was adjusted to match the turbidity of a MacFarland 0.5 standard. For each assay plate, 1.0 ml of the standardized inoculum was added to 225 ml of melted and cooled (52 °C) agar medium. The seeded agar was then mixed and poured into an assay plate located on a leveling table. Wells, 5.5 mm in diameter, were next cut and removed from the agar plates (normally, 81 evenly spaced wells were cut from each plate). The wells were then filled with the specimens or with standard solutions (ca. 100 µl). All standards or specimens were tested in three widely separated wells. After a 1-h prediffusion period at room temperature, the plates were incubated at 32 °C for 16–18 h. The zones of inhibition were then measured to the nearest 0.1 mm using calipers. For each specimen or standard, the mean of triplicate zone diameters was calculated. For each assay plate, a polynomial regression analysis was utilized to establish a dose-response curve for the standards. The concentration in each unknown sample was then calculated by applying the polynomial regression formula, following the principles outlined by Bennett et al. (7).

Figure 1 presents the dose-response curves for standards prepared in four different diluents; all were

tested on the same assay plate. When diluted in human milk, mouse plasma or water, the zone diameters were essentially identical. However, when diluted in human plasma, the zone diameters were reduced by 5 to 6 mm. Presumably, this was due to protein binding of roxithromycin at concentrations less than 32 µg/ml. The apparent lack of protein binding with mouse plasma is an interesting observation that we confirmed several times. For urine assays, the standards were prepared in 0.1 M Tris buffer rather than normal urine because urine is difficult to standardize. In Tris buffer, roxithromycin standards gave zones similar to those observed with water as the diluent. The pH of the diluent was not as critical as the pH of the agar medium.

Both urine and plasma assays consistently detected concentrations ≥ 1.0 µg/ml. In human plasma, concentrations of 0.5 µg/ml usually produced measurable zones. When testing mouse plasma, on the other hand, concentrations as low as 0.25 µg/ml were consistently detected. In ten human milk samples, levels of 0.25 µg/ml gave zone ≥ 12 mm in diameter. Nonspecific inhibition by all ten predose samples prevented interpretation of tests with concentrations lower than 0.25 µg/ml.

Table 1: Recovery of roxithromycin added to plasma, urine or human milk obtained from untreated volunteers.

Specimen	Expected recovery µg/ml	Quadruplicate bioassay results		
		Mean ^a	CV (%) ^b	Percentage of expected
Plasma	22.4	23.1	3.2	103.1
	11.2	9.5	4.5	84.8
	5.6	4.9	2.6	88.4
	2.8	2.8	6.3	98.2
	1.4	1.4	3.6	97.9
	0.7	0.7	1.9	98.6
Average			3.7	95.2
Urine	96	111.0	22.5	115.6
	48	48.2	6.6	100.4
	24	25.2	8.8	105.0
	12	12.4	7.6	103.3
	6	6.9	7.1	115.8
	3	4.0	13.5	133.3
	1.5	2.4	5.3	168.0
Average			10.2	120.2
Milk	24	25.0	6.6	104.2
	12	11.7	3.2	97.5
	6	5.0	17.1	83.3
	3	2.4	8.5	80.0
	1.5	1.5	11.2	100.0
	0.75	0.86	3.5	114.7
Average			7.35	96.6

^a Mean values expressed as µg/ml.
^b Coefficient of variation for quadruplicate tests.

Table 2: Recovery of roxithromycin added to plasma or urine specimens from human subjects receiving roxithromycin.

Specimen	Sample no.	Bioassay ($\mu\text{g/ml}$)		Recovery of roxithromycin	
		Unseeded	Seeded ^a	$\mu\text{g/ml}$	Percentage of expected ^a
Plasma	1	5.5	11.7	6.2	88.6
	2	6.1	11.7	5.6	80.0
	3	5.1	10.3	5.2	74.3
	4	3.9	11.1	7.2	102.9
	5	2.9	7.6	6.7	95.7
	6	1.3	9.5	8.2	117.1
Average				6.5	93.1
Urine	1	24	94	70	109.0
	2	23	96	73	114.0
	3	22	104	82	128.0
	4	22	83	61	95.0
	5	20	108	88	137.0
Average				74.8	116.9

^a Plasma samples were seeded with 7.0 $\mu\text{g/ml}$ and urine specimens with 64 $\mu\text{g/ml}$.

Eleven different batches of control plasma containing 6 μg roxithromycin per ml were tested over a 16-month period of time. The overall mean assay value for 491 separate tests was 5.6 $\mu\text{g/ml}$ and the average coefficient of variation was 7.6% (5.0%–12%). A low-concentration (2 $\mu\text{g/ml}$) control plasma sample was included in some of the studies over the 16-month period. The mean of 101 assay values was 2.1 $\mu\text{g/ml}$ and the average coefficient of variation was 12.3%. Six different batches of urine controls containing 10 $\mu\text{g/ml}$ were tested over the same period of time. The mean assay value for 216 separate tests was 9.97 $\mu\text{g/ml}$ and the average coefficient of variation was 10.9% (8%–13%). Control urine samples containing 25 μg roxithromycin per ml were tested 105 times: the mean value was 22.5 $\mu\text{g/ml}$ and the average coefficient of variation was 11%. Ten separate assays were performed with human milk seeded with 6 μg roxithromycin per ml. The mean value was 5.5 $\mu\text{g/ml}$ and the coefficient of variation was 9.8%.

Specimens collected before treatment with any antibiotic were seeded with varying concentrations of roxithromycin and then tested in quadruplicate. Table 1 lists the expected concentrations and mean of four assay values. Plasma, urine and milk assay values respectively averaged 95%, 120% and 97%, of the expected values.

To further assess the accuracy of the bioassay system, urine or plasma samples from subjects receiving roxithromycin were selected for additional recovery experiments. Each specimen was diluted with equal volumes of normal plasma or urine with or without additional roxithromycin. The seeded and unseeded samples were then tested by the appropriate bioassay system (Table 2). The plasma samples should have

demonstrated an additional 7.0 $\mu\text{g/ml}$ in the seeded sample. The average recovery was 6.5 $\mu\text{g/ml}$ (93% of that expected). The urine specimens were seeded with 64 $\mu\text{g/ml}$ and the average amount recovered was 74.8 $\mu\text{g/ml}$ (117% of that expected).

The accuracy and precision of roxithromycin bioassay procedures described here should be satisfactory for most clinical applications. In view of the increased activity of roxithromycin in an alkaline pH, Antibiotic Medium No. 11 (pH 8.0) was selected for the assays. Selection of *Sarcina lutea* (ATCC 9341) as the test strain was also influenced by a desire to maximize the sensitivity of the test system. Preliminary trials with spore suspensions of *Bacillus subtilis* (ATCC 6633 (Difco) showed *Sarcina lutea* to be at least five times more sensitive. The one-hour prediffusion period can be eliminated if a sensitivity below 1.0 $\mu\text{g/ml}$ is not required. The reduced potency that results from protein binding is compensated for by diluting standards in normal plasma from the same species. Binding to urinary sediment can present an important source of variability. Urine specimens must be thoroughly mixed before removing the samples. Although the clinical significance of the apparent binding to urinary sediments is unknown, it did influence our decision to prepare the standards in Tris buffer rather than urine from normal volunteers.

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