Semiautomated Turbidimetric Bioassay for the Ionophore A23187

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The inhibitory effect of A23187 against *Staphylococcus aureus* was used to quantitate the activity of this ionophore in fermentation samples and in isolation and crystallization samples. The assay was shown to be rapid and reproducible.

A23187 is a divalent cation ionophore obtained from a strain of *Streptomyces chartreusis* (R. L. Hamill et al., Prog. Abstr. Intersci. Conf. Antimicrob. Agents Chemother., 12th, Atlantic City, N.J., Abstr. 65, p. 32, 1972). The biological properties of A23187 were described previously and the structure was determined by X-ray crystallography (1). This ionophore selectively transports divalent cations across artificial or biological membranes (4, 7). It has been used to alter the intracellular Ca^{2+} concentrations in a wide variety of cells, tissues, and subcellular organelles (2, 5, 6). A23187 inhibits the growth of bacteria and fungi. The present study was initiated to quantitate A23187 activity in fermentation extracts and in various solutions derived from its isolation and purification. *Staphylococcus aureus* (H-Heatley strain, NRRL B-314) was used as the test organism. A stock cell suspension of *S. aureus* was prepared by growing the cells in nutrient broth and harvesting during the log phase of growth. The cells were centrifuged, suspended in glycerol-lactose, and maintained in the vapor phase of liquid nitrogen. The inoculum was used at a concentration of 3×10^5 colony-forming units per ml of assay medium.

The medium for the assay of A23187 has the following composition: peptone (Difco), 0.5%; yeast extract (Difco), 0.15%; beef extract (BBL), 0.15%; sodium chloride, 0.35%; K_2HPO_4 , 0.37%; and KH_2PO_4 , 0.13%. The pH of the medium was 7.0 before sterilization and remained essentially unchanged after the medium was autoclaved for 20 min at 121°C . Prior to use, the medium was chilled and supplemented to contain 0.1% glucose by using a sterile 50% glucose stock solution.

Crystalline A23187 (free acid) was used as the assay standard. A stock standard solution was prepared by dissolving crystalline A23187 in methanol to a final concentration of 1,000 $\mu\text{g}/\text{ml}$. Stock standard solutions were stable for

greater than 30 days when refrigerated. On the day of the assay, standard mixtures containing 0.4, 0.6, 0.8, and 1.0 μg of A23187 per ml were prepared by diluting the stock standard solution with a 1:1 water-methanol mixture. Unknown samples were prepared for assay by diluting in a 1:1 water-methanol mixture to ob-

FIG. 1. Typical dose-response curve of *S. aureus* to a range of A23187 concentrations.

TABLE 1. Precision of turbidimetric assays on seven representative samples of A23187

Samples	A23187 activity (μg/ml of sample solution)									Mean ^a	SD ^b	COV ^c (%)
	Day 1				Day 2				Day 3, test 1			
	Test 1	Test 2	Test 3	Test 4	Test 1	Test 2	Test 3	Test 4				
A	92	97	103	101	93	101	99	98	87	98	5.7	5.8
	93	102	100	103	94	103	106	107	90			
B	91	94	94	99	95	100	92	93	90	96	3.7	3.9
	98	95	101	94	97	97	104	96	92			
C	87	93	90	93	89	96	90	89	86	90	2.6	2.9
	90	89	93	90	91	91	94	91	87			
D	234	248	241	239	247	247	243	248	245	242	4.8	2.0
	240	274	233	240	244	242	235	236	242			
E	80	77	80	72	81	75	81	76	82	79	2.5	3.2
	79	82	80	79	80	80	80	79	79			
F	50	50	53	49	52	53	52	53	56	52	1.7	3.3
	51	54	51	50	51	53	52	53	53			
G	158	162	160	164	164	167	162	166	168	166	3.7	2.3
	172	162	168	165	167	166	171	169	168			

^a Mean = $\bar{X} = \sum \chi_i / n$, where χ_i = individual values, and n = number of individual values.

^b Standard deviation = $s = (\text{SD}) \sqrt{\sum (\chi_i - \bar{X})^2 / (n - 1)}$.

^c Coefficient of variation (COV) = $(s) (100\%) / \bar{X}$.

TABLE 2. A23187 assay characterization from analysis of variance results

Assay scheme		Resulting precision	
No. of days tested	No. of tests per day	Coefficient of variation (%)	Approximate 95% confidence limits (%)
1	1	2.71	± 5.4
1	2	2.12	4.2
1	3	1.88	3.8
1	4	1.75	3.5
2	1	1.93	3.9
2	2	1.51	3.0
2	3	1.34	2.7
2	4	1.24	2.6
3	4	1.02	2.0

tain about 0.75 to 0.85 μg of A23187 activity per ml. The assays were performed by using an Autoturb system, which provides a high order of precision for the microbiological assay of vitamins and antibiotics (3). Test solutions were transferred to an Autoturb Dilutor Carousel, and the dilutor was calibrated to take four 0.15-ml volumes of each solution. The test was incubated in a 37°C constant-temperature water bath. Transmittance was measured by an Autoturb Reader and expressed in terms of millivolts (1,000 mV equals 100% transmittance). Growth of the test organism was monitored by

readings taken from control tubes that received the 1:1 water-methanol diluent but no antibiotic. The test was terminated in about 4 h when control tubes had reached 45% transmission measured at 642 nm. The data from the Autoturb Reader were interfaced through an analogue to a digital converter and to a Thomas paper tape punch. The resulting tape was loaded into an external time-sharing computer for data analysis. Data were analyzed point to point by plotting transmittance versus concentration.

An example of the dose-response relationship of A23187 to *S. aureus* by using the described assay is shown in Fig. 1. To test the precision of the assay, representative samples from fermentation extracts and isolation samples were assayed over a period of 3 days. Replications of the tests were made on days 1 and 2, and replications of the samples within the tests were made on days 1, 2, and 3. The same standard dose-response solutions and sample solutions were used in all of the assays. Solutions A, B, and C are partially purified preparations from the isolation, and samples D, E, F, and G are representative extracts from the fermentation.

The test results are presented in Table 1 with summary statistics for each sample. The mean value is the overall average for each sample, and the standard deviation is a measure of the

spread of the individual results. The coefficient of variation is the standard deviation expressed as a percentage of the mean (see footnotes of Table 1 for equations).

An analysis of variance was run to determine the sources and magnitude of variation contributing to the overall assay variation. These results were then used to calculate a theoretical coefficient of variation for a given number of tests on a given number of days (the assay scheme). The approximate 95% confidence limits are merely two times the calculated coefficient of variation. The purpose of the results given in Table 2 is to promote an appropriate assay scheme to give a specified level of precision. If, for example, it is desired to detect a 4% change in potency at a 95% confidence level, then it is necessary to use an assay scheme that would yield a result with 95% confidence limits less than or equal to 4%. We can look in Table 2 and see that to achieve this precision we must make at least three assays on 1 day or one assay on each of 2 days to accomplish an overall estimation of potency with the desired precision.

The described assay procedure using semi-automated equipment was successfully used to quantitate A23187 activity in extracts derived from culture development studies and pilot plant fermentations, as well as to evaluate A23187 purification and crystallization procedures. Due to the worldwide research interest

in this ionophore, it is felt that the knowledge of the methodology for quantitation of the activity of this compound may be useful to other investigators. Although automated equipment was used in this study, adaptation of the procedure to manual turbidimetric assays would be relatively easy.

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