A NEW BIOASSAY SYSTEM OF SULFONAMIDE

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INTRODUCTION

Bioassay of sulfonamide is not always easy to perform routinely, because antimicrobial activities of the drug on various bacteria are not so high and constant as other antibiotics. Therefore the BRATTON-MARSHALL's chemical assay has been performed as a routine method to determine the drug concentration in body fluid. But bioassay is more desirable than chemical method to appreciate the clinical results of chemotherapeutics.

On the bioassay of sulfonamide, several methods have been reported. Among them the following two reports are more interesting and significant than any others, those are, OKAMOTO[1] reported the use of GLUCOSE-SIMONS medium and *Escherichia coli* K-12 and KANAZAWA *et al.*[2] reported the use of MUELLER-HINTON medium and *Staphylococcus aureus* 209P or *Escherichia coli* K-12.

These two methods, however, were not always satisfactory to perform routinely in our laboratory.

This paper deals with a new bioassay system in which thin layer cup method is applied to estimate sulfonamide by using a synthetic medium modified SAUTON's prescription, and spore suspension of *Bacillus subtilis* PCI 219 as a test microorganism. Applying this system, blood levels of sulfamethopyrazine (SMP), sulfisomezole (SIM), and sulfadimethoxine (SDM) after oral administration to adults with cross over design were determined.

MATERIALS AND METHODS

Preparation of test microorganisms

The cells of *Bacillus subtilis* PCI 219 cultured on nutrient agar at 37°C for 7 days were harvested and washed once with distilled water by centrifugation at 3,000 rpm for 30 minutes. After discarding supernatant fluid, the residue was resuspended with distilled water and heated twice at 65°C for 60 minutes every 24 hours. Then, the upper layer after centrifugation at 1,000 rpm for 5 minutes was used as original spores suspension. This suspension was adjusted to contain 3.2×10^6 spores/ml.

Preparation of medium

The basic medium consisted of aspartic acid 0.4%, citric acid 0.2%, diammonium citrate 0.005%, K_2HPO_4 0.05%, $MgSO_4\cdot7H_2O$ 0.05%, and glucose 0.4%. Glucose was autoclaved separately and added into the basic medium, and pH of the medium was adjusted to 7.0.

Bioassay

Thin layer cup method was applied. Five milliliters of the agar medium containing 2% of the spore suspension were poured into a petri-dish of 9 cm diameter, and one or two cups were placed on the agar plate, and each cup was filled with 0.067 M phosphate buffer solution (pH 7.0) containing sulfonamide. The agar plates were kept at 5°C for 3 hours in order to diffuse the drug before incubation. After incubation at 37°C for 16 to 18 hours, all cups were removed and 1 ml of 0.3% 2,3,5-triphenyltetrazoliumchloride solution poured onto the surface of every plate, and the plates were maintained at 37°C for 10 to 20 minutes. Then the staining solution was discarded, and diameter of inhibition zones were determined.

Chemical assay

The estimation of free form of sulfonamide was performed by using BRATTON-MARSHALL's method.

Estimation of blood level in human

One gram of sulfonamide was orally administrated as single dose to three adults with cross over design. The blood was taken out at appropriate intervals and the plasma was diluted with 0.067 M phosphate buffer to optimal concentration and supplied to assay.

RESULTS AND DISCUSSION

Bioassay

Table 1 shows the combination of various components to prepare synthetic media. Using these media, the inhibition zones by SMP were determined by the condition shown in Table 2.

The results in Table 2 indicate that the inhibition zone at a concentration of 1 mcg/ml of SMP was

Table 1. Components of synthetic media

Medium No.	Aspartic acid (%)	Citric acid (%)	Diammonium citrate (%)	Glucose (%)	Beef extract (%)	KH_2PO_4 (%)	$MgSO_4 \cdot 7H_2O$ (%)	Agar (%)
1	0.4	0.2	0.005	0.4	0.5	0.05	0.05	1.8
2	—	0.2	—	0.4	0.5	0.05	0.05	1.8
3	—	—	0.005	0.4	0.5	0.05	0.05	1.8
4	—	—	—	0.4	0.5	0.05	0.05	1.8
5	—	—	0.005	0.4	0.5	—	0.05	1.8
6	—	0.2	0.005	0.4	0.5	0.05	0.05	1.8
7	0.4	0.2	—	0.4	0.5	0.05	0.05	1.8
8	0.4	—	0.005	0.4	0.5	0.05	0.05	1.8
9	0.4	0.2	0.005	0.4	0.2	0.05	0.05	1.8
10	0.4	0.2	0.005	0.4	0.5	—	—	1.8

Table 2. Inhibition zone by sulfonamide in various media

Medium No.	Diffusion time (%)	Incubation time (hour)	Bacterial inoculum size (%)	Diameter of inhibition zone* (mm)	
				Concentration of SMP (mcg/ml) 10	1
1	3	18	2	23.00	14.00
2	3	18	2	31.25	—
3	3	18	2	28.75	—
4	3	18	2	22.50	—
5	3	18	2	28.00	—
6	3	18	2	27.50	—
7	3	18	2	23.75	—
8	3	18	2	23.50	—
9	3	18	2	23.50	—
10	3	18	2	23.25	—

* Spore suspension of *Bacillus subtilis* PCI 219 was used as a test microorganism.

Table 3. Inhibition zone by sulfonamide in the medium No. 1 in absence of beef extract

Diffusion time (hour)	Incubation time (hour)	Bacterial inoculum size (%)	Diameter of inhibition zone* (mm)				
			Concentration of SMP (mcg/ml) 5	2.5	1.25	0.63	0.31
3	18	2	40.00	32.50	26.25	21.50	—
2	18	2	36.50	28.50	20.00	—	—
3	16	2	40.25	34.75	29.25	23.00	—
3	16	0.5		37.50	30.00	25.00	19.00

* Spore suspension of *Bacillus subtilis* PCI 219 was used as a test microorganism.

found in the use of medium No. 1 alone. Therefore, it was found that medium No. 1 was particularly suitable for the bioassay of sulfonamide.

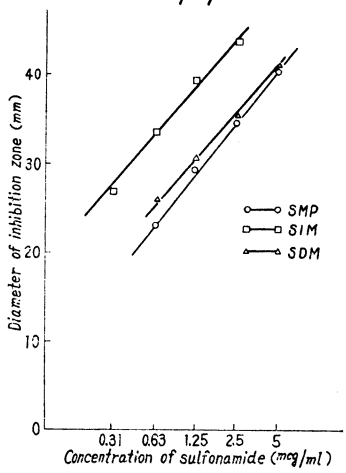
From the medium beef extract was taken out to enlarge the diameter of inhibition zone at lower concentration than 1 mcg/ml of the drug and various conditions such as diffusion time, incubation time, and bacterial concentration in the medium were investigated. These results were shown in Table 3.

When the bacterial concentration in the medium is 0.5%, the inhibition zone was more enlarged than in the case of 2%, but surrounding area of a zone was rather poor in clearness to estimate exactly the size of diameter.

Summarizing these results, it was decided that the following conditions, the use of medium No. 1 in absence of beef extract, 3 hours for diffusion, 16 hours in incubation, and 2% of bacterial inoculum, were the most suitable for a new bioassay system. According to this method, constant results were obtained with repeated experiments.

Fig. 1 shows the standard curves of SMP, SIM, and SDM in a new bioassay system.

Fig. 1 Standard curves of sulfonamides with a new bioassay system



Blood level

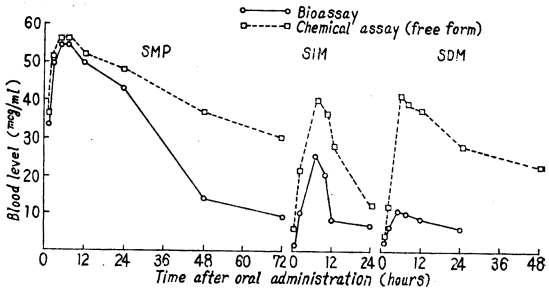
Using the standard curves of Fig. 1, the blood levels after single oral administration of 1 g of the three sulfonamides in adults were determined. The results were shown in Fig. 2, being compared with those of chemical assay.

The peaks of blood levels were found at 5 to 6 hours after administration, and the peak-levels in the bioassay were SMP: 55 mcg/ml, SIM: 25 mcg/ml, and SDM: 11 mcg/ml, while those in the chemical assay were SMP: 56 mcg/ml, SIM: 40 mcg/ml, and SDM: 41 mcg/ml.

As shown in Fig. 2, the results of chemical assay showed higher blood level than those of bioassay. The same fact was already reported by SAKAI *et al.*[3] or KANAZAWA *et al.*[2], and they considered that this discrepancy might be caused by the protein binding property of sulfonamide in blood.

From the above results, it may be said that the new bioassay system described in this report can

Fig. 2 Blood levels of sulfonamides, being compared with bioassay and chemical assay



be employed as a routine assay method, and it may be an essential tool to discuss pharmacokinetics of sulfa drugs in body.

SUMMARY

A new bioassay system to estimate sulfonamide was investigated. A thin layer cup method by using a synthetic medium modified SAUTON's prescription, and spore suspension of *Bacillus subtilis* PCI 219 as a test microorganism was found.

Applying this bioassay, blood levels of three sulfonamides were determined, being compared with BRATTON-MARSHALL's chemical assay.

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