Colorimetric bioassay for measuring aminoglycoside antibiotics in blood spots on filter paper

—Individual application to netilmicin, gentamicin and isepamicin—

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A simplified microbiological detection method for aminoglycoside antibiotics, using *Bacillus subtilis* and thymolphthalein as colorimetric indicator, is described. In each test, netilmicin, gentamicin and isepamicin were sufficiently recovered from individual blood spots during incubation in the same medium. After incubation, the concentration of blue-colored thymolphthalein was determined by both fluorescence polarization immunoassay and a spectrophotometer equipped with a multichannel photodiode array detector for each aminoglycoside. There was proportionality between the concentration of each aminoglycoside and the degree of discoloration of thymolphthalein. No difference was found between these two methods. The advantage of this method is that the approximate individual concentration of netilmicin, gentamicin or isepamicin can also be detected visually by using a paper disc containing thymolphthalein for the individual aminoglycoside as follows: navy blue, more than peak concentration; light blue, within therapeutic range; white, less than trough concentration, and ready for injection.

Key words: colorimetric bioassay, respirometric bioassay, netilmicin/gentamicin/isepamicin, *Bacillus subtilis*, blood spots

INTRODUCTION

Aminoglycosides (AGs) have been extensively used for treatment of serious infections with gram-negative bacteria. The widespread use of these antibiotics has led to the development of methods to quantify them in pharmaceutical preparations and biological fluids. Treatment with AGs is associated with several adverse effects, and drug monitoring is effective in preventing ototoxic and nephrotoxic adverse effects and confirming therapeutic concentrations because of their narrow therapeutic range.

Microbiological procedures, traditionally time-consuming, have been widely used and theoretically accepted for detection of AGs and are based on the antimicrobial activities of these drugs. In other words, microbiological procedures still have many advantages if the analysis can be accomplished within an adequate time.

We have been investigating the colorimetric bioassay of netilmicin (NTL)[1,2], an aminoglycoside. Discoloration of the color indicator, thymolphthalein (TP), on a paper disc (TP-indicator disc) is due to the presence of CO_2 produced by *Bacillus subtilis*.

Sample collection by finger pricking instead of venipuncture is potentially useful for determining the level of AGs in pediatric patients[3] where venipuncture is often difficult or on some occasions impossible. Although the applicability of the blood spot method[3] is limited, it has an advantage in terms of sample collection. In view of these advantages, there is a need to develop an assay combining a colorimetric bioassay and the blood spot method for AGs. We determined the applicability of a proposed method by using NTL dissolved in buffer. To avoid any interference with biological components, different specimens were evaluated prior to application to the other antibiotics.

In the present study, first we investigated interference by blood components in CO_2 production, then determined, by colorimetric bioassay, the individual concentration of NTL, gentamicin sulfate (GM) or isepamicin sulfate (ISP) in blood collected from the ear vein of a rabbit by the blood spot method. Application of the bioassay during therapy to confirm trough aminoglycoside blood levels before subsequent injection is extremely useful for preventing adverse effects.

MATERIALS AND METHODS

Animals

Male rabbits weighing about 3 kg were used (Shimizu Animal Laboratories, Kyoto, Japan). The rabbits were acclimatized in a temperature (24℃) and humidity (60%, relative humidity) controlled room before treatment.

Chemicals, equipment and others

The chemicals, fluorescence polarization immunoassay (FPIA, TDx®) equipment and other factors including the microbiological procedures used were as described previously[1~4] unless otherwise mentioned. GM and ISP were from Schering-Plough (Osaka, Japan), and a computerized spectrophotometer equipped with a flexible optical-fiber sensing probe and a photodiode array detector was from Otsuka Electronics (model MCPD-1000, Osaka).

Determination of CO_2 production with blood specimen

To determine the influence of blood components on CO_2 production, various concentrations of NTL ($0\sim10\,\mu$g/ml) were put into buffer, plasma, whole blood or blood spots and incubated under the condition of colorimetric bioassay[2]. CO_2 production was measured by gas chromatography (GC)[2].

Preparation of blood spots

The blood spots were prepared by spotting 100 μl of the standard solution or by adsorption to the blood sampling paper blood containing each aminoglycoside after administration of the aminoglycoside to rabbits, then stored at ambient temperature without contamination.

Recovery and reproducibility

To determine the rate of recovery of AGs from the blood spots, we prepared the blood spots containing NTL ($0\sim20\,\mu$g/ml). The blood spots was cut with scissors into five or six pieces, and then were placed in a Reacti-flask® containing 1 ml of nutrient broth and 2 ml of 0.1 M phosphate buffer solution (PBS) (pH8.0), and then placed in a water-bath (37±1℃) for 3 h. The extract from the blood spots was transferred to a Minicent® and centrifuged at 3,000×g for 15 min to remove hemoglobin which affects the FPIA assay. The filtrate was then concentrated by the conventional freeze-dry method to a range suitable for FPIA because the extract is too highly diluted with nutrient broth and PBS to be analyzed by FPIA directly. The filtrate reconstituted with 0.5 ml of the dilution buffer for TDx® was examined by FPIA.

Administration of antibiotics and blood sampling

Two experiments were performed with six rabbits, and were divided into three groups. The rabbits were fixed on a rabbit holder (Type: Oshida, Natsume, Tokyo). Each rabbit in the first group received NTL solution equivalent to 4 or 8 mg of NTL per kg by intramuscular injection. The rabbits in the other two groups received GM solution equivalent to 4 or 6 mg of GM per kg, or ISP solution equivalent to 12.5 or 20 mg of ISP per kg in the same manner. Blood was collected from the ear vein of each rabbit by means of a disposable syringe at the following times: before administration and 10 and 30 min and 1, 2, 4 and 6 h after the injection. Approximately 100 μl of the blood was also placed on blood sampling paper at the same time, and the blood spots were treated in the same manner as reported previously[2]. To determine the serum concentrations of AGs, the blood collected with the syringe was centrifuged at 3,000 rpm for 10 min, then assayed by FPIA to evaluate the data generated from the colorimetric bioassay presented in this report.

Spectrophotometric analysis of indicator disc

The appearance of the TP-indicator disc was checked visually and conventional color photographs of the disc were taken just after incubation with the blood spots. To prevent the discoloration phenomenon, we took photographs in a CO_2-free atmosphere by aeration using barium hydroxide solution. Then, to determine the rate of discoloration of the TP-indicator disc, the intensity of the blue color of the disc on photographs were determined at 655 nm as reflect

absorption wave length of the TP, by using the computerized spectrophotometer described above. The flexible optical-fiber sensing probe equipped the spectrophotometer is essential for measurement because neither TP-disc nor photographs are transparent, so a general spectrophotometer is not adequate for those specimens.

According to the Z 8729 of Japan Industrial Standards, the ΔEab^* (color change parameter) value was also calculated by the spectrophotometer as follows:

$\Delta Eab^* = [(\Delta a^*)^2 + (\Delta b^*)^2 + (\Delta L^*)^2]^{1/2}$ where a^* and b^* are the chromatically diagram and L^* is lightness.

RESULTS

Influence of blood components on CO_2 production

As the result of the influence of blood components on CO_2 production, the standard calibration curves for each specimen were as follows:

0.1 M PBS (pH 8.0): $y=4.76*e\hat{}(-0.206x)$ $r=0.970$,

plasma: $y=6.823*e\hat{}(-0.213x)$ $r=0.990$,

whole blood: $y=7.781*e\hat{}(-0.179x)$ $r=0.987$.

CO_2 production was slightly enhanced in the presence of both plasma and whole blood when compared with 0.1 M PBS (pH 8.0) as control samples. The results indicate that the difference in CO_2 production is not negligible for the colorimetric bioassay, so that an increase in NaOH concentration in the TP-indicator disc as adsorbent of CO_2 was required. As a result of the test, 50 μl of 0.8 M NaOH for plasma assay and 0.85 M for blood assay were adopted in this assay. The precision and SD for 0.1 M PBS (pH 8.0), plasma and blood are shown in Table 1.

Table 1. Influence of the blood components on the specimen for respirometric assay of netilmicin

Specimen	Concentration of NTL (μg/ml)	Respiratory CO_2 production* ($\times 10^{-6}$ mol) Intra-day	Inter-day
0.1 M PBS (pH 8.0)	0	6.07±0.11	5.99±0.07
	2.5	2.47±0.20	2.50±0.05
	5.0	1.30±0.08	1.39±0.09
	10.0	0.72±0.08	0.75±0.05
Plasma	0	7.79±0.23	8.03±0.13
	2.5	3.86±0.27	4.03±0.08
	5.0	1.91±0.03	1.83±0.02
	10.0	0.91±0.05	0.98±0.04
Whole blood	0	8.78±0.30	9.02±0.27
	2.5	4.59±0.12	4.13±0.17
	5.0	2.82±0.11	2.31±0.14
	10.0	1.41±0.16	1.32±0.07

*Mean±SD (n=3)

NTL: netilmicin

Recovery of NTL from the blood spots under incubation conditions

In our early study[8], we evaluated solvents for extracting AGs from the blood spots, and we chose 0.5 M Na_2HPO_4 as the eluant of AGs. However, we could not use that eluent in this study because of the increased pH and ion strength of the incubation medium. Therefore, to ensure recovery of NTL from the blood spots in the incubation medium consisted of 0.1 M PBS, we determined NTL in the elute by FPIA. The results demonstrated that the incubation medium was satisfactory for eluting NTL from the blood spots under the conditions used, although hemoglobin was also released from the blood spots (Table 2).

Calibration curves of CO_2 production for AGs

The CO_2 production profiles of blood spots containing NTL, GM or ISP during incubation was confirmed by GC and are shown in Fig. 1. We observed an excellent linear correlation between the concentration of these antibiotics and the logarithm of CO_2 production, indicating that the eluate from the blood spots containing sufficient amounts of aminoglycoside for quantitation, in other words, elution of aminoglycoside from the blood spots, are sufficient under the incubation conditions. The calibration curves for NTL, GM and ISP in blood spots were linear over the range of concentrations effective against each aminoglycoside. The calibration curves provided for this study were as follows (Fig. 1):

NTL: $y=6.618*e\hat{}(-0.141x)$ $r=0.986$,

GM: $y=6.927*e\hat{}(-0.168x)$ $r=0.996$,

ISP: $y=7.518*e\hat{}(-0.048x)$ $r=0.982$.

Table 2. Recovery of netilmicin from blood spots by fluorescence polarization immunoassay (TDx™)

NTL concentration added (μg/ml)	NTL concentration found* (μg/ml)	Recovery (%)
0	ND**	–
2.5	2.10±0.12	88.0
5.0	4.17±0.12	83.4
10.0	8.44±0.31	84.4
20.0	18.48±0.63	92.4

*Mean±SD (n=3)

**Not detected

NTL: netilmicin

Spectrophotometric analysis of indicator disc

The rate of discoloration of the TP-indicator disc was determined visually. To confirm the rate of discoloration by comparing with visual results, we used a spectrophotometry for quantitative evaluation of the disc. As shown in Fig. 2 (a), a good linear correlation was observed between NTL concentration and reflect absorbance by the spectrophotometric method. We also found that

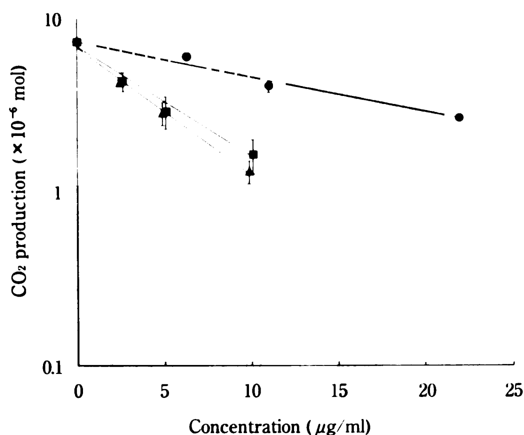

Fig. 1. Respiratory CO_2 production by *Bacillus subtilis*.
● netilmicin, ▲ gentamicin, ■ isepamicin
Each value represents the means±SD (N=3).

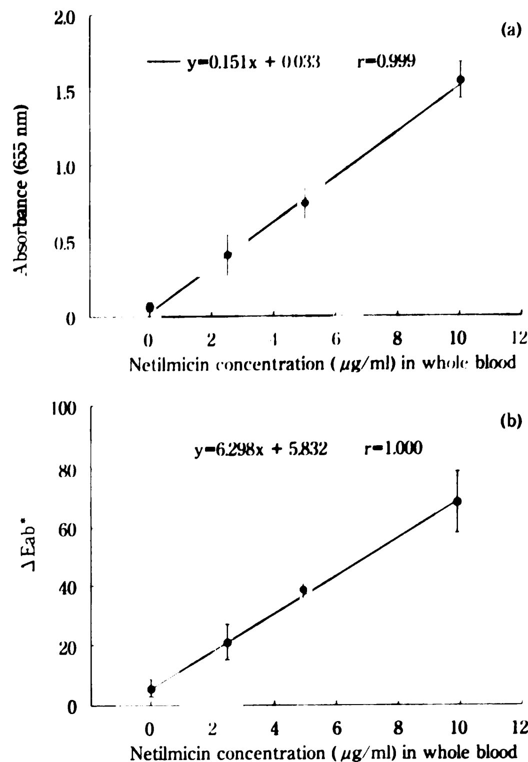

Fig. 2. Relationship between netilmicin concentration and absorbance (a), and ΔEab* (b) on TP-indicator disc.
Each value represents the means±SD (n=3).

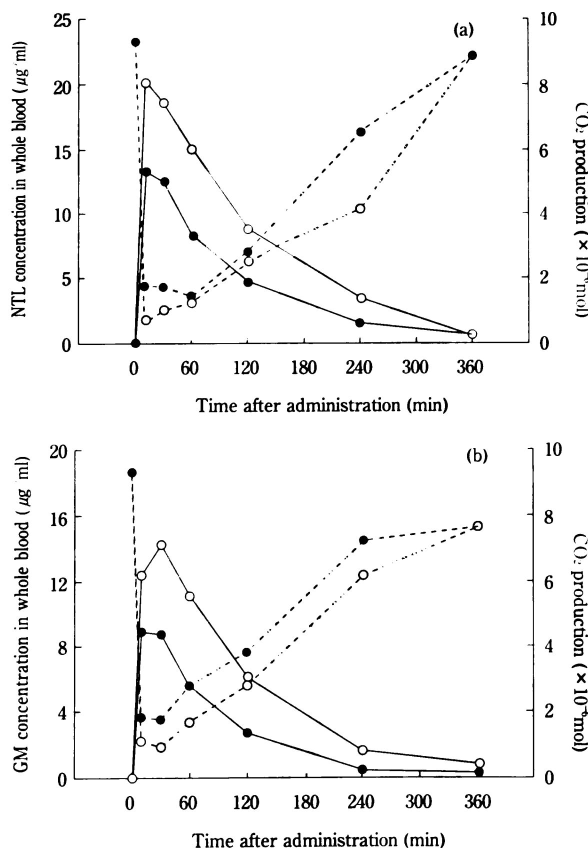

Fig. 3. Time profiles of aminoglycoside antibiotics concentration and CO_2 production after intramuscular administration to rabbits.
a) NTL; b) GM; c) ISP. Solid lines, Concentration; Each value represents the means±SD (n=1). Dashed lines, CO_2 production; Each value represents the means±SD (n=3). Dose: closed symbols, 4 mg/kg for NTL and GM; 12.5 mg/kg for ISP, open symbols, 8 mg/kg for NTL; 6 mg/kg for GM, and 20 mg/kg for ISP.
NTL: netilmicin, GM: gentamicin, ISP: isepamicin

NTL concentration versus ΔEab* value had a linear correlation (y=6.298x+5.832, r=1.000) [Fig. 2 (b)].

Blood concentration of AGs

As shown by the solid lines in Fig. 3, the individual concentrations of AGs in blood and the amount of CO_2 produced, were measured, respectively, by FPIA and GC, as one of the conventional assay methods, using blood spots as the specimen after intramuscular administration. AGs were rapidly absorbed from the injection site of each rabbit, and reached peak concentration about 15 to 30 min after administration.

In contrast, CO_2 production by *B. subtilis* was definitely inhibited in the presence of AGs and the TP-indicator disc showed no discoloration. This means that the aminoglycoside concentration in the blood reached around peak level. The time versus aminoglycoside concentration profile and time versus CO_2 production profile showed symmetrical results (Fig. 3), indicating that the colorimetric bioassay can be applied as a range assay method for detection of levels lower than trough or therapeutic range levels, or more than peak levels (Table 3).

This colorimetric bioassay also can be applied for other AGs, GM and ISP. NTL and GM has the same MIC (≤0.19 μg/ml) against *B. Subtilis*[6]. On the other hand, ISP has the double MIC (0.2 μg/ml) of NTL (0.1 μg/ml) against *B. Subtilis*[7].

Table 3. Individual time profiles for netilmicin, gentamicin and isepamicin for concentration, ΔEab*, visual appearance and estimated range

AGs	Dose (mg/kg)	Time (min)	Concentration in whole blood (μg/ml)	ΔEab*	Visual appearance	Estimated range
NTL	4	10	13.3	72	Navy blue	More than peak
		30	12.5	77	Navy blue	More than peak
		60	8.3	69	Navy blue	More than peak
		120	4.6	40	Light blue	Therapeutic range
		240	1.5	5	White	Less than trough
		360	0.6	1	White	Less than trough
	8	10	20.4	77	Navy blue	More than peak
		30	18.6	76	Navy blue	More than peak
		60	15.0	73	Navy blue	More than peak
		120	8.8	69	Navy blue	More than peak
		240	3.4	24	Light blue	Therapeutic range
		360	0.6	4	White	Less than trough
GM	4	10	9.0	73	Navy blue	More than peak
		30	8.8	71	Navy blue	More than peak
		60	5.6	44	Light blue	Therapeutic range
		120	2.8	14	Light blue	Therapeutic range
		240	0.5	3	White	Less than trough
		360	0.2	2	White	Less than trough
	6	10	12.4	72	Navy blue	More than peak
		30	14.4	73	Navy blue	More than peak
		60	11.1	72	Navy blue	More than peak
		120	6.2	52	Light blue	Therapeutic range
		240	1.7	5	White	Less than trough
		360	0.6	2	White	Less than trough
ISP	12.5	10	15.1	63	Light blue	Therapeutic range
		30	18.4	67	Light blue	Therapeutic range
		60	16.8	65	Light blue	Therapeutic range
		120	8.7	38	Light blue	Therapeutic range
		240	2.3	4	White	Less than trough
		360	0.9	3	White	Less than trough
	20	10	21.2	74	Navy blue	More than peak
		30	28.4	80	Navy blue	More than peak
		60	24.7	76	Navy blue	More than peak
		120	16.3	63	Light blue	Therapeutic range
		240	7.5	27	Light blue	Therapeutic range
		360	1.4	3	White	Less than trough

NTL: netilmicin, GM: gentamicin, ISP: isepamicin

DISCUSSION

In Japan, since the launching of gentamicin more than 20 years ago, AGs have continued to be used extensively in the treatment of serious gram-negative bacterial infections. Therapeutic activity seems to be directly dose-dependent and related to the ratio of the peak plasma concentration to minimal inhibitory concentration. On the other hand, severe adverse effects have been observed with AGs. Nephrotoxicity and ototoxicity have been reported to occur with high serum concentrations. The intensity of toxic effects also seems to be associated with the area under the curve. In this situation, drug monitoring is required to ensure adequate drug concentrations in the serum and to avoid potentially toxic accumulation.

Traditional microbiological assays for antibiotics are based on the inhibition of growth of a sensitive microorganism. The assays provide a measurement of the true biological activity of antibiotics, whereas nonmicrobiological methods measure concentration chemically. However, the traditional microbiological assays require a long incubation time and expertise in handling the microorganism under aseptic conditions. In view of these difficulties, several methods have been reported[8,9] for bioassays to reduce testing time and improve procedures.

In recently published studies we demonstrated that an aminoglycoside, NTL, can be measured with a suspension of *B. subtilis* and a colorimetric TP-indicator disc. The method uses the principle that the antibiotic inhibits CO_2 production by the bacterial cells and the CO_2 produced is absorbed into an alkaline solution containing TP as the colorimetric indicator.

Samples on filter paper have been used for assay of many blood components, and microbiological assay has also been used for various antibiotics, but this is the first report, to our knowledge, of application of the combination to NTL, GM or ISP assay by using blood spots as one of the biological specimens. This simple and convenient method of assay shows excellent correlation between the concentration of each aminoglycoside in blood and discoloration of the indicator disc.

The proposed method also has another advantage because discoloration from navy blue to white (the original color of the paper disc) is based on the amount of CO_2 produced by *B. subtilis*. We can therefore measure antibiotic levels in blood visually without any equipment as follows: navy blue, more than peak level; light blue, therapeutic level; white, less than trough level and ready for injection.

To prevent systemic drug accumulation, before repeated injection during therapy with the AGs it will be necessary to reconsider the dosing regimen when the blue color remains on the TP-indicator disc.

In conclusion, the colorimetric bioassay associated with the blood spot method described above is a reliable method for measuring the concentration of NTL, GM or ISP in the blood. It is particularly appropriate for confirming the trough level prior to repeated administration to patients.

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REFERENCES

1) Tsuda Y, Wakamatsu H, Fujimoto T: Studies on a colorimetric bioassay for netilmicin using an indicator disc. Chemotherapy 41: 641～648, 1993
2) Tsuda Y, Wakamatsu H, Yoshizawa E, Kitagawa E, Fujimoto T: Microbiological determination of netilmicin using a thin-layer chromatography scanner. J. J. Antibiotics 46: 1～7, 1993
3) Fujimoto T, Tsuda Y, Tawa R, Hirose S: Fluorescence polarization immunoassay of gentamicin in blood spotted on filter paper. Chemotherapy 37: 578～582, 1989
4) Kase K, Shoshihara M, Tsuda Y, Yoshizawa E, Fujimoto T: Determination of isepamicin in the eluates from the dried blood spots on filter paper for monitoring of blood levels in a guinea-pig. Yakugaku Zasshi. 109: 938～942, 1989
5) Fujimoto T, Tawa R, Hirose S: Fluorometric determination of sisomicin, an aminoglycoside antibiotic, in dried blood spots on filter paper by reversed-phase high-performance liquid chromatography with pre-column derivatization. Chem. Pharm. Bull. 36: 1571～1574, 1988
6) Goto S, Ogawa M, Tsuji A, Kaneko Y, Kuwahara S: Bacteriological evaluation of netilmicin. Chemotherapy 29 (S-3): 25～40, 1981
7) Yamaji S, Mitsuhashi S, Iyobe S: Antibacterial activity of HAPA-B. Chemotherapy 33 (S-5): 1～21, 1985
8) Fraticelli Y M, Meyerhoff M E: On-line gas dialyzer for automated enzymatic analysis with potentiometric ammonia detection. Anal. Chem. 55: 359～

364, 1983
9) Simpson D L, Kobos R K: Potentiometric microbiological assay of gentamicin, streptomycin and neomycin with a carbon dioxide gas-sensing electrode. Anal. Chem. 55: 1974～1977, 1983

ろ紙採血法を用いるアミノ配糖体系抗生物質の微生物学的比色分析法

—ネチルマイシン，ゲンタマイシン，イセパマイシンへの応用—

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Bacillus subtilis を用いた微生物学的な方法と，チモールフタレインを呈色指示薬として用いる化学的な反応を組み合わせた，アミノ配糖体系抗生物質の簡便な測定法の開発とその確立を目的として検討を行った。アミノグリコシド系抗生物質として，ネチルマイシン（NTL），ゲンタマイシン（GM）またはイセパマイシン（ISP）を用いて，それぞれのろ紙採血法を用いた試料からの回収について検討を行った。その結果，NTL 水溶液で検討した同一の条件下，NTL，GM，ISP を含むろ紙からのそれぞれの溶出も十分であり，これらを別々に含む生体試料への適用も可能であることが確認できた。チモールフタレインを含むろ紙の青色と抗生物質の量の関係を，FPIA 法とフォトダイオードアレイ検出器を備えた多波長分光光度計を用いて確認した結果，十分な定量性を有していることが確認できた。したがって，目視によってチモールフタレインの青色が無色（ろ紙の白色）へと変化する度合いを以下のように観察すれば，試料中の抗生物質の量を簡便に測定することが初めて可能となった。

濃青色: 有効治療域濃度以上
淡青色: 有効治療域内濃度
無色（白色）: トラフ濃度以下，継続投与可

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