5-Fluorouracil (5-FU) is one of the most commonly used chemotherapeutics in the treatment of malignancies originating from breast, prostate, ovarian, skin and gastrointestinal tissues. Around 80% of administered dose of 5-FU is catabolized by dihydropyrimidine dehydrogenase (DPD). Patients, in whom a deficiency or insufficient activity of this enzyme is observed, are at great risk of development of severe, even lethal, 5-FU toxicity. According to recent studies, so far over 30 mutations of DPYD gene, which are associated with DPD deficiency/insufficiency, have already been discovered. Currently, there are several analytical methods used for measurements of DPD activity. However, in this paper we report a novel, simple, economical and more accessible spectrophotometric method for measurements of DPD activity in the peripheral blood mononuclear cells (PBMCs) that was developed and validated on analysis of 200 generally healthy volunteers aged 22–63. We present two spectrophotometric protocols in this study, and as a reference method we used already described reverse phase high-performance liquid chromatography (RP HPLC) analysis. Basing on our findings, we conclude that spectrophotometric methods may be used as a screening protocol preceding 5-FU-based chemotherapy. Nevertheless, before introduction into clinical reality, our results should be confirmed in further larger studies.

**Key words**: 5-fluorouracil, cancer, chemotherapy, dihydropyrimidine dehydrogenase, reverse phase high-performance liquid chromatography, spectrophotometric method
(ASPCR), restriction fragment length polymorphism (RFLP), and pyrosequencing (51-53). These methods offer rapid and high-throughput screening capabilities; however, they can only be used to screen for the known mutations. 5-FU pharmacogenetic studies have aided in the advancement of predicting patients’ response to 5-FU chemotherapy through the development of both phenotypic and genotypic assays for DPD (54).

DPD activity is found in most tissues (55-59). Several clinical studies suggest that over-expression of DPD in tumor tissue is associated with poor response to 5-FU-based therapy (60). DPD activity is not only important for establishment of 5-FU pharmacokinetics and clinical toxicity, but also is a critical factor in determination of the availability of 5-FU for anabolism to active metabolites within the tumor (61, 62). This raises the possibility that measuring the level of DPD within a tumor itself may have predictive value in prognosis whether a tumor is likely to respond to 5-FU (63). The role of intra-tumor DPD activity was evaluated in the clinical setting. For head and neck cancer patients, DPD activity was detectable in all tumor samples. Patients with complete response to 5-FU-based induction chemotherapy exhibited lower tumoral DPD activities than partial or non-responding individuals (64). Fuke et al. reported that DPD mRNA levels correlate with DPD enzyme activity (65). A quantitation of DPD mRNA by reverse transcription-PCR is an alternative method for determining both - DPD concentration or activity in samples where is insufficient amount of tissue to perform a DPD enzyme assay (63). The use of biochemical assays requires availability of clinical samples (e.g. needle biopsies) that weight minimum 10–30 mg (66). The question whether routine prospective DPD testing for 5-FU-related severe toxicity should be generally recommended, and whether such testing should be rather based on a genetic testing or a functional test of DPD activity, which may more comprehensively account for non-genetic factors affecting DPD activity, cannot be definitely answered at present (29).

So far, several analytical methods for measurements of DPD activity have been described. These are either genetic tests detecting single nucleotide polymorphisms in DPYD gene and/or its mRNA expression (1, 2, 8, 38-40, 47, 67-72), or biochemical methods analyzing metabolites of 5-FU catabolic pathway (uracil levels in plasma/urine or in breathing test; coefficients of dihydrouracil/uracil or dihydrothymine/thymine concentration, as well as, measurements of 2-fluoro-β-alanine levels (1, 6, 11, 13, 26, 38, 39, 46, 71, 73-75). In humans, verification of DPD activity is generally performed on the basis of its activity in peripheral blood mononuclear cells (PBMCs), that is lymphocytes and monocytes (6, 11, 13, 26, 38, 46). In their study, Etienne et al. used 14C-fluorouracil as a substrate in reaction catalyzed by DPD, in which a product - 14C-5-dihydrofluorouracil is generated, and they measured it using reverse phase high-performance liquid chromatography (RP-HPLC) (76). According to this method mean DPD activity was 0.222 nmol/min/mg protein (ranging from 0.065 to 0.559 nmol/min/mg protein). The mean value in this study was close to that reported by Lu et al. (0.189 nmol/min/mg protein) (77). The other method, in which 4-14C-thymine is used as a substrate for DPD, is also developed. In this method DPD activity is calculated on the basis of 4-14C-dihydrothymine levels, measured by RP-HPLC (8, 38, 78). Both of these possess a significant limitation associated with a need for use of isotope-labeled standards (46). Deporte-Fetty et al. developed the method, in which 5-FU was used as a substrate, and the level of 5-FUH2 (metabolite generated in reaction catalyzed by DPD) was measured using RP-HPLC (11). Van Kuilenburg et al. for detection of DPD insufficiency were using thymine, and in this analysis dihydrothymine levels were measured using HPLC-MS/MS (13).

Clinical introduction of diagnostic methods that would allow for a direct or indirect measurements of DPD activity in plasma and in lymphocytes, would enable to predict whether 5-FU therapy is potentially toxic for a patient. Various methods available today for determination of DPD status on a phenotypic basis were developed in a cost- and time-effectiveness perspective. Spectrophotometric methods offer functional testing that requires basic analytical apparatus, such a standard spectrophotometer, quick sample preparation steps with affordable reagents and decent run-times, making them cheap and easier to adapt in most laboratories. Hence, the aim of this study was to validate a novel spectrophotometric method, for measurements of DPD activity, that would be simple and easy to introduce into clinical reality for screening purposes among group of patients qualified for 5-FU chemotherapy.

MATERIAL AND METHODS

Mononuclear cells were isolated from peripheral blood (PBMC) samples derived from 200 healthy volunteers (102 female and 98 male), aged 22–63 years (mean 34±13). Included individuals were generally healthy subjects with no chronic diseases, as well as, neglected presence of any acute diseases for at least 1 month prior to inclusion in the study. Moreover, none of the patients has been on any pharmacotherapy for at least 2 weeks before the study. Peripheral venous blood samples (15–20 mL) were collected and mixed with K2EDTA. The study protocol was approved by the Bioethical Committee of the Pomeranian Medical University in Szczecin, and all patients provided informed written consent prior to participation.

Isolation of peripheral blood mononuclear cells from peripheral blood

The blood was centrifuged (250 g; 10 min; 20°C). Received plasma was transferred to a fresh tube, and stored in –80°C until the assays were performed. PBMCs were isolated using Lymphoprep™ Tubes (Axis-Shield, Norway), and the PBMCs numbers were counted by a hemocytometer. The number of isolated cells was 0.49±10^5–0.13±10^6 PBMC/µl. Purity of PBMC was assessed by morphologic examination of a cell suspension stained using Jenner-Giema method on a cytopsin. Granulocytes were not present in the fraction containing the PBMC (<2%).

Assays of dihydroxypropylamine dehydrogenase activity in peripheral blood mononuclear cells using spectrophotometric methods

Cellular lysates were obtained by using Complete Lysis-M, EDTA-free reagents (Roch Diagnostics GmbH, Germany). During analysis all reagents and lysates were maintained in 37°C temperature. Measurements were performed using UV-VIS spectrophotometer (Spectord 250; Analytic Jena, Germany). DPD activity was determined by two methods (Fig. 1). Method I assayed DPD activity by monitoring the oxidation of NADPH+H+. The assay for DPD activity monitored spectrophotometrically at 340 nm (Fig. 1) the decrease in absorbance associated with the oxidation of NADPH+H+ by thymine. A reaction mixture was contained PBMC lysate, 35 mmol/l K2HPO4, 2.5 mmol/l MgCl2•H2O, 1 mmol/l dithiothreitol, 250 µmol/l NADPH+H+, 200 µmol/l cardumine, 50 mmol/l ceruline, 10 µmol/l mevastatin, 25 µmol/l thymine, pH 7.4. Value of NADPH+H+ molar extinction coefficient ε340nm = 6.22×10^3 L•mol•1•cm−1) was applied during calculations.

Method II monitored DPD activity by the absorbance increase at 260 nm associated with oxidation of 5,6-
Cellular lysates were obtained by using Complete Lysis-M, EDTA-free reagents (Roche Diagnostics GmbH, Germany). During analysis all reagents and lysates were maintained in 37°C temperature. PBMCs lysates were mixed with 35 mmol/l K2HPO4, 2.5 mmol/l MgCl2•H2O, 1 mmol/l dithiothreitol, 750 µmol/l NADP+, 25 µmol/l 5,6-dihydrouracil, 250 µmol/l NADPH+H+, and 25 µmol/l uracil (pH 7.4). After 20th, 40th, 60th and 80th minute reaction was inhibited by addition of Na2SO4 solution and acidification with 2 mol/l H2SO4. Afterwards 25 µmol/l 5-fluorocytosine (internal standard) was added and mixture was centrifuged (10,000 g, 10 minutes; 20°C). Received supernatants were stored in –80°C until until the assays were performed.

Samples were defrosted at room temperature and centrifuged (5000 g; 10 minutes; 20°C). Supernatants were analyzed by HPLC. The assay was developed using a high-performance liquid chromatograph Hewlett Packard 1100 Series (Agilent Technologies, Warsaw, Poland). Chromatographic data were processed by HP Chemstation software (Agilent Technologies, Warsaw, Poland).

Reversed-phase HPLC was performed on SymmetryShield™ RP18 column (250 mm × 4.6 mm, 5 µm) (Waters, Warsaw, Poland). The column temperature was 25°C. A isocratic method was used where KH2PO4 buffer was used as a moving phase. The flow rate was 0.8 mL/min. 50 µL samples were injected every 30 minutes. Detection wavelength was 260 nm for uracil and 5-fluorocytosine (internal standard) and 210 nm for 5,6-dihydrouracil. The quantitation was based on peak areas with internal standard calibration.

Validation procedure of spectrophotometric methods for analysis of dihydropyrimidine dehydrogenase activity in peripheral blood mononuclear cells

Validation of novel methods was conducted through performance of characteristics of spectrophotometric methods, comparison of them with a reversed phase high-performance liquid chromatography (RP-HPLC) method, as well as, through verification of applicability of these methods as a screening tools allowing for detection of lower/insufficient DPD activity in PBMC. The following parameters were determined: limit of detection (LOD), limit of quantitation (LOQ), linearity, accuracy, precision (intra-assay and inter-assay). The validation procedure was performed using a purified DPD samples from PBMCs. Nominal activities were verified by HPLC-MS. LOD value was determined using a signal-to-noise ratio of 3:1, whereas the LOQ was defined as the lowest concentration that could be measured within 80–120% of the nominal value and with a coefficient of variation below 10%. To determine the accuracy and precision of the assay four level of DPD control samples (purified extract of this enzyme) were obtained: two low levels of 0.08 nmol/min/mg protein, 0.16 nmol/min/mg protein, medium level of 0.3 nmol/min/mg protein, and high level of 0.62 nmol/min/mg protein. The nominal value of DPD activity in control samples were verified by HPLC-MS. The accuracy was defined as a percentage of a ratio of the observed activity to the nominal activity. Intra-assay and inter-assay precisions were defined as a percentage of the ratio of the standards deviation to the mean (coefficient of variation). For intra-assay precision and accuracy 10 control samples of each concentration were measured on the same day. For inter-assay assessment all control samples were measured on each of 10 days.

The reverse phase high-performance liquid chromatography (RP HPLC) assay was cross-validated by the analysis of PBMC DPD activity obtained from 25 subjects using RP HPLC analysis of 5FUH2 formed from 5FU (11) and uracil. Statistical comparisons of data resulting from cross-validation experiments were performed using the paired t-test and regression analysis.

Statistical analysis

Received results were subjected to a statistical analysis. To determine the distribution of variables, Shapiro-Wilk’s test was used, which results demonstrated the lowest normal distribution of analyzed variable. Methods were characterized and presented by such parameters as: arithmetical mean, standard deviation, median value, quartile range, as well as, its upper and lower values. Differences between analyzed parameters were compared using t-Student’s, and ANOVA tests. In order to verify the diagnostic precision of analyzed methods, these were subjected to further verification using receiver operating characteristics (ROC) curves, as well as, such parameters as sensitivity, and specificity characterizing each method were calculated. Statistical analysis was performed using STATISTICA PL v. 10.1 (Statsoft, Krakow, Poland) statistical analysis software. Statistical significance was defined when p values were less than 0.05.

RESULTS

Received results demonstrated that limit of detection (LOD) values were of 0.016±0.0014 and 0.0128±0.0011 nmol/min/mg protein for spectrophotometric I and II, respectively (Table 1). LOD for reverse phase high-performance liquid chromatography (RP HPLC) was 0.0074±0.0014 nmol/min/mg protein, and was significantly lower than those of spectrophotometric methods (p<0.05). Limit of quantitation (LOQ) values for method I and II were 0.0531±0.0031 nmol/min/mg protein and 0.0516±0.0027 nmol/min/mg protein (respectively), as well as, again were proved to be statistically higher (p<0.05) than the LOQ values of RP HPLC (0.0484±0.0014 nmol/min/mg protein).

Intra-assay and inter-assay precisions and accuracy of the methods were evaluated using four levels of dihydropyrimidine dehydrogenase (DPD) control samples (0.08, 0.16, 0.30, 0.62 nmol/min/mg protein). Intra-assay precision for method I was 6.6, 2.4, 1.8 and 2.0%, respectively for four levels tested (Table 2). Respective accuracies were 119, 108, 112 and 103%. Intra-assay precision for method II was 5.6, 2.7, 4.5 and 2.6%, respectively for four levels tested. Respective accuracies were 114, 108, 109 and 102%. Calculated coefficients of intra-assay variation for RP HPLC analysis were 4.8, 2.7, 2.0 and 2.5%. The accuracies for all levels were 101, 99, 99 and 98%.

Inte-assay precision for the lowest level of a control sample were 4.9, 3.1 and 8.3, respectively for method I, II and RP HPLC. Respective accuracies were 117, 110, 100% (Table 3). Inter-assay precision for the second level of a control sample (0.16 nmol/min/mg protein) were 2.0, 3.7 and 3.2, for method I, II and RP HPLC (respectively). Respective accuracies were 109,
Table 1. Limit of detection (LOD) and quantitation (LOQ) of examined methods of dihydropyrimidine dehydrogenase (DPD) activity measurements in peripheral blood mononuclear cells (PBMCs) (nmol/min/mg protein). S.D. - standard deviation; C.V. - coefficient of variation.

Table 2. Intra-assay precision and accuracy of examined methods of dihydropyrimidine dehydrogenase (DPD) activity measurements in peripheral blood mononuclear cells (PBMCs) (nmol/min/mg protein). S.D. - standard deviation; C.V. - coefficient of variation.

Table 3. Inter-assay precision and accuracy of examined methods of dihydropyrimidine dehydrogenase (DPD) activity measurements in peripheral blood mononuclear cells (PBMCs) (nmol/min/mg protein). S.D. - standard deviation; C.V. - coefficient of variation.

Table 4. Dihydropyrimidine dehydrogenase (DPD) activity in peripheral blood mononuclear cells (PBMCs) (nmol/min/mg protein) in 200 volunteers - basic statistical analysis.

The linearity for all methods was observed in the protein concentration range of 0.15–4.0 mg/mL. The values of basic statistical parameters, calculated for DPD activity in PBMCs in 200 volunteers, for all analyzed methods are presented in Table 4. Mean DPD activity in the I and II spectrophotometric method was 0.57 and 0.53 nmol/min/mg (respectively), whereas median values were of 0.58 and 0.51 nmol/min/mg (respectively). In the first spectrophotometric
method the minimal concentration was of 0.21 nmol/min/mg protein, while in the second - 0.30 nmol/min/mg protein. As a consequence also differences in the lower and upper quartile were also observed, although the quartile range was 0.31 nmol/min/mg protein for both spectrophotometric methods. In RP HPLC method mean DPD activity was of 0.30±0.17 nmol/min/mg protein, its minimal activity was of 0.06 nmol/min/mg protein, and quartile range - 0.27 nmol/min/mg protein.

Analyzed laboratory methods were also compared by verification of the dispersion of received results (Figs. 2 and 3). Twenty five samples were collected from healthy subject and assayed for DPD activity using both analyses. In this comparison the method, in which RP HPLC analysis of 5-fluorodihydoruracil (5FUH2) generation from 5-FU was verified, was designated as the reference procedure, whereas the method described in the present paper was the test method. The mean ratio (test method/reference method) was 1.00 with a coefficient of variation of 4.3%. The paired t-test showed no significant difference between both analytical methods (p=0.76). The regression curve is depicted on Fig. 4.

Application of a cut-off value estimated by RP HPLC at 0.155 nmol/min/mg protein level, allowed for prediction of occurrence of 5-FU side effects with 98% sensitivity and 97% specificity. In case of spectrophotometric methods, application of a cut-off limit of 0.345 nmol/min/mg protein (method I) or of Table 5. Diagnostic accuracy of analyzed methods in dihydropyrimidine dehydrogenase (DPD) activity measurements. AUC - area under ROC curve; 95% CI - confidence interval of AUC value; p - level of significance.

<table>
<thead>
<tr>
<th>Method</th>
<th>AUC</th>
<th>95%CI</th>
<th>p</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP HPLC</td>
<td>1.00</td>
<td>0.852-1.00</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>spectrophotometric I</td>
<td>0.956</td>
<td>0.779-0.999</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>spectrophotometric II</td>
<td>0.978</td>
<td>0.813-1.00</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

Table 6. Analysis of diagnostics accuracy of analyzed methods in dihydroxypyrimidine dehydrogenase (DPD) activity measurements.

<table>
<thead>
<tr>
<th>Method</th>
<th>Cut-off value [nmol/min/mg]</th>
<th>Sensitivity (95%CI)</th>
<th>Specificity (95%CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RP HPLC</td>
<td>0.155</td>
<td>1.00</td>
<td>0.478-1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>1.00</td>
<td>0.815-1.00</td>
</tr>
<tr>
<td>spectrophotometric I</td>
<td>0.345</td>
<td>0.8</td>
<td>0.284-0.995</td>
</tr>
<tr>
<td>spectrophotometric II</td>
<td>0.338</td>
<td>1.00</td>
<td>0.478-1.00</td>
</tr>
<tr>
<td></td>
<td></td>
<td>0.944</td>
<td>0.727-0.999</td>
</tr>
</tbody>
</table>

Table 5. Diagnostic accuracy of analyzed methods in dihydropyrimidine dehydrogenase (DPD) activity measurements.

PBM cells
  Complete Lysis-M
    PBMC lysates
      35 mM K2HPO4
      2.5 mM MgCl2·H2O
      1 mM dithiothreitol
      pH 7.4
Method I
Inhibitors:
200 µM carmustine
50 mM cerulenine
10 µM mewastatin
+ 250 µM NADPH+H+
25 µM thymine
λ340 nm
ε340 nm 6.22·10³ L·mol⁻¹·cm⁻¹

Method II
750 µM NADP⁺
25 µM 5.6-dihydrouracil
λ 260 nm
ε260 nm 6.5·10³ L·mol⁻¹·cm⁻¹

Fig. 1. Spectrophotometric methods – scheme.
Fig. 2. Comparison between the reverse phase high-performance liquid chromatography (RP HPLC) method and spectrophotometric method I.

Fig. 3. Comparison between the reverse phase high-performance liquid chromatography (RP HPLC) method and spectrophotometric method II.
0.338 nmol/min/mg protein (method II) resulted in 80% and 98% sensitivity, as well as, in 97% and 94% specificity (for method I and II respectively) (*Tables 5 and 6*).

**DISCUSSION**

Due to relatively high toxicity of 5-FU-based chemotherapy it seems relevant to introduce screening tests prior to administration of this drug. According to the recent studies lack or insufficient DPD activity seems to be responsible for occurrence of a vast majority of side effects associated with 5-FU therapy (1-3, 5, 6, 46). Hence, detection of such improper DPD function could allow for prediction of 5-FU intolerance, and application of alternative chemotherapeutics or could be a tool for estimation of initial doses of 5-FU in patients with DPD deficiency (79).

DPD is expressed in several organs and tissues, however, its highest activity is observed in the liver and PBMCs (12, 26, 38, 80, 81). This last compartment of DPD activity seems to be of greatest importance, as according to some studies a strong correlation between DPD activity in PBMCs and systemic 5-FU clearance is observed (13, 26, 48). Moreover, in around 3–5% of general population a partial deficiency in this enzyme’s activity is observed, and in 0.1% complete lack of DPD activity is diagnosed (46, 82).

Currently, it is believed that the most simple, economical and accessible method of analysis of DPD activity in PBMCs would be spectrophotometry. Lohkam *et al.* were making such studies by measurements of reduction of absorption at 340 nm wavelength associated with catabolism of uracil to 5,6-dihydrouracil (83).

So far two spectrophotometric methods for measurements of DPD activity have been developed, and these were validated and compared with a RP-HPLC method. In the first method a reduction in absorption at 340 nm was observed, and this was associated with decreasing levels of NADPH+H+, required for reaction in which thymine is reduced to dihydrothymine. DPD is not the only enzyme that requires NADPH+H+, as also other cellular enzymes, such as glutathione reductase, HMG-CoA reductase, and/or fatty acid synthase may be responsible for use of this cofactor. In order to avoid potential interaction between these enzymes in reaction catalyzed by DPD, various inhibitors were used, and these included carbustine (inhibitor of glutathione reductase) (84), cerulenin (inhibitor of fatty acids’ synthase) (85), and mevastatin (HMG-CoA reductase inhibitor).

The second method was concentrated on measurements of increase in absorption at 260 nm wavelength, caused by increasing levels of uracil that was generated in reaction of dihydrouracil oxidation associated with NADP+ reduction. These measurements were performed in PBMCs lysates. In both methods dithiothreitol was used as an activator of DPD, and was increasing its activity 3-fold (86).

Estimated detection and quantitation limits for both spectrophotometric methods were significantly higher than those observed for RP-HPLC. Intra- and inter-assay precision was analyzed on the basis of calculated coefficients of variation. Precision of spectrophotometric methods is comparable to this of RP-HPLC, however, their accuracy is low in comparison to RP-HPLC.

Values of DPD activity in PBMCs derived from healthy volunteers are significantly higher when measured using spectrophotometry. This probably reflects matrix effect caused by determination of DPD activity in PBMC lysates without purification procedure. High values of correlations’ coefficients between analyzed methods, as well as, analysis of dispersion of received results suggest, that spectrophotometric methods may
To date, various threshold limits for DPD activity have been proposed to identify patients with a risk of developing severe 5-FU associated toxicity (13). However, a threshold of DPD activity in PBMCs, below which patients could be at higher risk, was not defined because DPD activity was measured by several laboratories using different techniques. However, since patients with major or partial DPD-deficiency had DPD activity value \(\leq 30\%\) of the normal mean in the general population (below the lower limit of the 95% distribution range) (38, 87-90) in PBMC tests, this value (of approximately 0.15 nmol/min/mg protein), could be considered as a cut-off for patients at higher risk (46, 11). Critical analysis of ROC curves allowed for verification of diagnostic value of examined methods. Application of a standard RP-HPLC cut-off limit value of 0.15 nmol/min/mg protein (11) allowed for division of analyzed group of healthy individuals into a subgroups with lowered and normal DPD activity (11).

In order to verify the predictive potential of spectrophotometric methods for detection of abnormal DPD activity (and thereby the risk of occurrence of 5-FU - related side effects), careful analysis of the diagnostic value of these methods with graphical analysis of ROC curves were performed. In our study application of a cut-off value of 0.345 nmol/min/mg protein allowed for achievement of 80% sensitivity and 97% specificity in method I, while in method II a cut-off value of 0.338 nmol/min/mg protein was used and resulted in 98% sensitivity and 94% specificity. In terms of screening methods much attention is mainly paid to their diagnostics sensitivity (91). In our study the method II actually met this criterion. However, even though we feel that in the clinical setting such result received by using spectrophotometry still should be confirmed by RP-HPLC and/or genetic tests.

In summary, we conclude that spectrophotometric methods may be used as a screening tool for analysis of DPD activity in humans, however, for their introduction in the clinical screening preceding 5-FU-based chemotherapy further studies on larger cohorts of patients are undoubtedly needed. In our opinion these should also include patients with improper DPD activity confirmed by genetic analyses.

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