A Simple Method for the Characteristic Differentiation of Antibiotics by TLC-Bioautography in Graded Concentration of Ammonium Chloride

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ABSTRACT

A simple classification method for 24 known antibiotics by TLC-bioautographic procedure was developed. The approach used was to change the Rf values in seven TLC systems with an ammonium chloride solution in a graded concentration range (0.5, 1, 2, 3, 5, 10 and 20%). The antibiotics were divided into four groups (A to D) showing the characteristic behavior of Rf values corresponding to similarities in chemical structure: B-lactam, aminoglycoside, macrolide and tetracycline antibiotics. TLC-bioautography helps to estimate the character of antibiotics and the characteristic change of Rf values may be very useful for classifying unknown residual antibiotics in animal samples as a routine laboratory test.

The extensive usage of antimicrobial agents in animal husbandry may lead to problems with residues in food hygiene and public health. Much effort has been put into the detection and determination of residual antimicrobial agents in animal organ tissues and body fluids (7,9,10). However, most of these reports were limited largely to a few major antibiotics. In practice, identification tests on animal samples have to be made on unknown residual antibiotics and much time is needed to obtain their complete identification. If the definite characteristic pattern of an antimicrobial agent can be found in early tests, the following approach to identification becomes easy and fast.

Although there are many antibiotics available for veterinary use, these can be grouped on the basis of similarities in chemical structure, such as β-lactam, tetracycline, macrolide and aminoglycoside antibiotics.

Thin-layer chromatography (TLC) is one of the simplest and quickest methods for the identification of organic compounds. The identification of antibiotics by TLC has been described in a number of papers (3,4,5,10). On the other hand, Miyazaki et al. (6) and Uri (8) have reported that some antibiotics can be grouped according to their salting-out chromatograms with paper chromatography. The present paper summarizes studies on TLC-bioautography using 24 known antibiotics. The approach used was to change the Rf values in seven TLC systems with an ammonium chloride solution in a graded concentration range. The antibiotics to be analyzed were divided into four groups (A to D) according to similarities in chemical structure. This simplified method may identify unknown residual antibiotics in animal husbandry as a routine laboratory procedure.

MATERIALS AND METHODS

Antibiotics used

Twenty-four known antibiotic standards (Table 1) were obtained from chemical suppliers or directly from pharmaceutical companies. All these antibiotics were of known potency and aqueous solutions were prepared at suitable concentrations before use.

TLC plate used

Plates with 0.25 mm silica gel 60 (20 × 20 cm) without fluorescent indicator (Merck Co.) were used after cutting to a size of 1.5 × 18 cm.

Developing solvent

Seven different concentrations of ammonium chloride (0.5, 1, 2, 3, 5, 10 and 20%) in water were used as the developing solvent for each antibiotic.

Test organisms

These were Bacillus subtilis ATCC 6633 (B. subtilis) and Micrococcus luteus ATCC 9341 (M. luteus). B. subtilis was added to the culture medium to give a bacterial count of 1.3 x 10^9/ml from a spore suspension prepared by the method of Smither et al. (7). M. luteus was added to the culture medium at a concentration of 0.5%, using fresh cultures propagated in nutrient broth at 37°C for 18 h.

Culture media

Agar medium consisted of (g/L): polypeptone (Daigo Chemical Co., Ltd., Osaka, Japan), 5; beef extract (Difco), 3; bacto-agar (Difco), 18; for B. subtilis; polypeptone, 10; beef extract, 5; sodium chloride, 2.5; bacto-agar, 18; for M. luteus. The pH of the medium was adjusted to pH 6.0 with hydrochloric acid.
Application to residues in tissue

The bovine kidney tissue (10 g), previously shown to be antibiotic-free, was inoculated with 50 mcg/g with penicillin-G. The extract from the tissue was subjected to the TLC-bioautographic method using seven solvent systems.

The tissue was homogenized threefold with methanol and centrifuged at 900 G for 10 min. The resultant supernatant was acid for *B. subtilis* and to pH 8.0 with sodium hydroxide for *M. luteus*.

**TLC-bioautographic method**

Five microliters of a graded concentration of each antibiotic were spotted by microsyringe onto 7 TLC plates and each plate was developed in the upper layer at room temperature using 7 different concentrations of NH₄Cl, respectively. When the front of the solvent reached 15 cm, the plate was removed from the developing tube (1.8 x 20 cm) of the chromatographic chamber and air-dried with a fan. The dried TLC plates were each placed on an agar plate seeded with test organism for 2 h at room temperature. After the TLC plates had been removed from the agar plate, they were incubated overnight at 37°C and Rf values were measured.

**Measurement of detection limits**

Five microliters of a graded concentration of each antibiotic was applied in a similar manner to that described above and the TLC plates were developed ascendingly with 3% NH₄Cl solution as the developing solvent. The minimum amount of each antibiotic showing an inhibition clear zone on the agar medium was taken as the detection limit.

**Application to residues in tissue**

The bovine kidney tissue (10 g), previously shown to be antibiotic-free, was inoculated with 50 mcg/g with penicillin-G. The extract from the tissue was subjected to the TLC-bioautographic method using seven solvent systems.

The tissue was homogenized threefold with methanol and centrifuged at 900 G for 10 min. The resultant supernatant was

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**TABLE 1. Changes of Rf values in TLC-bioautography with ammonium chloride solution of graded concentration.**

<table>
<thead>
<tr>
<th>Family</th>
<th>Antibiotics</th>
<th>Test organism</th>
<th>0.5</th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>5</th>
<th>10</th>
<th>20</th>
<th>Minimum amount detected (µg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Penicillins</td>
<td>Penicillin-G</td>
<td><em>M. luteus</em></td>
<td>0.81</td>
<td>0.71</td>
<td>0.69</td>
<td>0.67</td>
<td>0.59</td>
<td>0.47</td>
<td>0.33</td>
<td>0.0020</td>
</tr>
<tr>
<td></td>
<td>Ampicillin</td>
<td><em>M. luteus</em></td>
<td>0.68</td>
<td>0.68</td>
<td>0.67</td>
<td>0.67</td>
<td>0.65</td>
<td>0.64</td>
<td>0.56</td>
<td>0.0078</td>
</tr>
<tr>
<td></td>
<td>Cloxacillin</td>
<td><em>M. luteus</em></td>
<td>0.56</td>
<td>0.48</td>
<td>0.42</td>
<td>0.37</td>
<td>0.33</td>
<td>0.26</td>
<td>0.21</td>
<td>0.0313</td>
</tr>
<tr>
<td></td>
<td>Oxacillin</td>
<td><em>M. luteus</em></td>
<td>0.74</td>
<td>0.60</td>
<td>0.51</td>
<td>0.47</td>
<td>0.40</td>
<td>0.31</td>
<td>0.20</td>
<td>0.0078</td>
</tr>
<tr>
<td></td>
<td>Sulbenicillin</td>
<td><em>M. luteus</em></td>
<td>0.90</td>
<td>0.90</td>
<td>0.89</td>
<td>0.80</td>
<td>0.73</td>
<td>0.58</td>
<td>0.44</td>
<td>0.0625</td>
</tr>
<tr>
<td></td>
<td>Methicillin</td>
<td><em>M. luteus</em></td>
<td>0.51</td>
<td>0.49</td>
<td>0.43</td>
<td>0.30</td>
<td>0.24</td>
<td>0.20</td>
<td>0.12</td>
<td>0.1250</td>
</tr>
<tr>
<td>Cephalosporins</td>
<td>Cefazolin</td>
<td><em>M. luteus</em></td>
<td>0.39</td>
<td>0.31</td>
<td>0.25</td>
<td>0.25</td>
<td>0.20</td>
<td>0.17</td>
<td>0.13</td>
<td>0.0313</td>
</tr>
<tr>
<td></td>
<td>Cefoxitin</td>
<td><em>M. luteus</em></td>
<td>0.83</td>
<td>0.77</td>
<td>0.68</td>
<td>0.58</td>
<td>0.55</td>
<td>0.48</td>
<td>0.39</td>
<td>0.0313</td>
</tr>
<tr>
<td></td>
<td>Cephalotin</td>
<td><em>M. luteus</em></td>
<td>0.68</td>
<td>0.54</td>
<td>0.46</td>
<td>0.42</td>
<td>0.37</td>
<td>0.36</td>
<td>0.20</td>
<td>0.0020</td>
</tr>
<tr>
<td></td>
<td>Cephaloglycin</td>
<td><em>M. luteus</em></td>
<td>0.50</td>
<td>0.49</td>
<td>0.49</td>
<td>0.46</td>
<td>0.46</td>
<td>0.41</td>
<td>0.32</td>
<td>0.0020</td>
</tr>
<tr>
<td>Aminoglycosides</td>
<td>Streptomycin</td>
<td><em>B. subtilis</em></td>
<td>0.06</td>
<td>0.10</td>
<td>0.24</td>
<td>0.47</td>
<td>0.87</td>
<td>1.0</td>
<td>1.0</td>
<td>1.25</td>
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<tr>
<td></td>
<td>Dihydrostreptomycin</td>
<td><em>B. subtilis</em></td>
<td>0.02</td>
<td>0.05</td>
<td>0.18</td>
<td>0.39</td>
<td>0.66</td>
<td>0.85</td>
<td>0.89</td>
<td>1.25</td>
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<tr>
<td></td>
<td>Kanamycin</td>
<td><em>B. subtilis</em></td>
<td>0.02</td>
<td>0.02</td>
<td>0.03</td>
<td>0.05</td>
<td>0.17</td>
<td>0.62</td>
<td>0.88</td>
<td>1.25</td>
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<tr>
<td></td>
<td>Fradiomycin</td>
<td><em>B. subtilis</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.03</td>
<td>0.30</td>
<td>0.76</td>
<td>2.50</td>
</tr>
<tr>
<td></td>
<td>Gentamicin</td>
<td><em>B. subtilis</em></td>
<td>0</td>
<td>0</td>
<td>0.01</td>
<td>0.02</td>
<td>0.07</td>
<td>0.43</td>
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<td>0.625</td>
</tr>
<tr>
<td>Macrolides</td>
<td>Erythromycin</td>
<td><em>M. luteus</em></td>
<td>0.05</td>
<td>0.08</td>
<td>0.09</td>
<td>0.09</td>
<td>0.09</td>
<td>0.07</td>
<td>0.03</td>
<td>0.0156</td>
</tr>
<tr>
<td></td>
<td>Leucomycin</td>
<td><em>M. luteus</em></td>
<td>0.06</td>
<td>0.07</td>
<td>0.07</td>
<td>0.08</td>
<td>0.08</td>
<td>0.06</td>
<td>0.04</td>
<td>0.0313</td>
</tr>
<tr>
<td></td>
<td>Clindamycin</td>
<td><em>M. luteus</em></td>
<td>0.13</td>
<td>0.18</td>
<td>0.20</td>
<td>0.23</td>
<td>0.20</td>
<td>0.18</td>
<td>0.15</td>
<td>0.0625</td>
</tr>
<tr>
<td></td>
<td>Oleandomycin</td>
<td><em>M. luteus</em></td>
<td>0.08</td>
<td>0.08</td>
<td>0.12</td>
<td>0.12</td>
<td>0.11</td>
<td>0.08</td>
<td>0.05</td>
<td>0.0625</td>
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<tr>
<td></td>
<td>Tylosin</td>
<td><em>M. luteus</em></td>
<td>0.05</td>
<td>0.08</td>
<td>0.10</td>
<td>0.11</td>
<td>0.11</td>
<td>0.05</td>
<td>0.03</td>
<td>0.0625</td>
</tr>
<tr>
<td>Tetracyclines</td>
<td>Tetracycline</td>
<td><em>M. luteus</em></td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.05</td>
<td>0.05</td>
<td>0.06</td>
<td>0.25</td>
</tr>
<tr>
<td></td>
<td>Oxytetracycline</td>
<td><em>M. luteus</em></td>
<td>0.21</td>
<td>0.23</td>
<td>0.26</td>
<td>0.27</td>
<td>0.31</td>
<td>0.34</td>
<td>0.36</td>
<td>0.125</td>
</tr>
<tr>
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<td>Dimethylichlorotetracycline</td>
<td><em>M. luteus</em></td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.03</td>
<td>0.05</td>
<td>0.05</td>
<td>0.125</td>
</tr>
<tr>
<td></td>
<td>Methacycline</td>
<td><em>M. luteus</em></td>
<td>0.03</td>
<td>0.03</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.04</td>
<td>0.06</td>
<td>0.0625</td>
</tr>
</tbody>
</table>

**Figure 1. Classification of antibiotics by means of TLC-bioautography into four groups (A-D). A, β-lactams (penicillins and cephalosporins); B, aminoglycosides; C, macrolides; D, tetracyclines.**

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RESULTS AND DISCUSSION

Rf values in seven solvent systems of 24 antibiotics obtained by the TLC-bioautographic method are listed in Table 1. The characterization of each antibiotic family according to changes of Rf value was recognized.

According to the influence of the salt concentration on Rf values, the antibiotics were divided into four groups which corresponded to similarities in chemical structure. The four groups (A to D; see Fig. 1) are defined as follows.

Group A includes the β-lactam antibiotics, penicillins and cephalosporins. The highest Rf is in 0.5% salt solution and increasing concentrations of the salt cause a decrease in Rf values. The Rf values give S-shaped curves.

Group B includes aminoglycoside antibiotics, with increasing Rf values in response to increasing concentration of the salt, giving S-shaped curves of a reverse character.

Group C includes macrolide antibiotics. The Rf values increase with rising concentrations of salt and the Rf reaches a maximum at a salt concentration of 3 - 5%; at higher salt concentrations it decreases again, but does not reach zero.

Group D includes tetracycline antibiotics. The Rf values are almost unaffected by salt concentration, giving a nearly straight line parallel with the starting points.

The detection limit for each antibiotic is shown in Table 1. The limits ranged from 0.002 to 2.5 μg. The lowest were for β-lactam antibiotics, with values ranging from 0.002 to 0.125 μg.

TLC-bioautogram in the extract from bovine kidney tissue added with penicillin-G is illustrated in Fig. 2. The Rf values in each ammonium chloride solution were almost the same with those of standard penicillin-G solution and showed an S-shape curve.

Two organisms, B. subtilis and M. luteus, were used for developing the bioautographs in this study. Both organisms are used routinely for developing paper or thin-layer chromatograms and also for standard microbiological assays. The aminoglycoside antibiotics have been shown to be more highly sensitive to B. subtilis than to any other test organism for the detection of antibiotic residues, especially at pH 8.0 rather than pH 6.0 (9,10).

The salting-out chromatogram of an antibiotic is obtained by chromatography on a series of TLC plate strips. For the development of the strips by the ascending technique using seven solvent systems, increasing concentrations of ammonium chloride (0.5 to 20%) in water were used. The antibiotics were divided into four groups (A to D) showing the characteristic behavior of Rf values corresponding to similarities in chemical structure, β-lactam, aminoglycoside, macrolide and tetracycline antibiotics. Betina (1) and Betina and Nemec (2) reported that each antibiotic tested could be divided into four types of pH chromatogram according to the Rf values on paper strips impregnated with buffers in the range pH 2-10 as the mobile phase.

This TLC-bioautographic method using seven solvent systems as ascending concentration of salt is a simple and fast way to obtain Rf values. TLC-bioautography helps to estimate the character of antibiotics and the characteristic change of Rf values may be very useful for classifying unknown residual antibiotics in animal samples as a routine laboratory test. Subsequent tests to provide definite identification can be carried out easily by methods such as high-performance liquid chromatography or TLC using organic mobile phases suitable for the particular family of antibiotics. Use of the method also assists in the identification of unknown antibiotics in the pure state or when mixed with other biological materials.

REFERENCES


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Davis et al., con’t. from p. 777


