Identification and Determination of Oxytetracycline, Tiamulin, Lincomycin, and Spectinomycin in Veterinary Preparations by Thin-Layer Chromatography/Densitometry

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A thin-layer chromatographic/densitometric method was developed for the identification and quantitation of oxytetracycline, tiamulin, lincomycin, and spectinomycin in veterinary preparations. Silica gel-coated thin layer chromatography plates and 2 mobile phases were used to separate these constituents. The appropriate compositions of the suitable mobile phases were established: 10% citric acid solution–n-hexane–ethanol (80 + 1 + 1, v/v) and n-butanol–ethanol–chloroform–25% ammonia (4 + 5 + 2 + 5, v/v). Along with $R_I$ values and spot colors, direct UV and visual densitometric measurements were used for identification. Similar measuring ranges were used for quantitative analysis to obtain repeatable and reliable results for the preparations examined. The results of the quantitative analysis are characterized by a small confidence interval and are close to the declared contents of active constituents: oxytetracycline 30.01–0.38 g at $\lambda = 350$ nm and 30.24–0.86 g at $\lambda = 430$ nm; tiamulin, 10.19–0.86 g at $\lambda = 450$ nm; lincomycin, 2.27–0.08 g at $\lambda = 278$ nm; and spectinomycin, 2.18–0.07 g at $\lambda = 421$ nm. The recoveries for all antibiotics ranged from 100.01 to 102.54%.

Oxytetracycline, tiamulin, lincomycin, and spectinomycin are antibiotics that differ from each other in chemical composition, but they have similar physical and chemical properties such as solubility in water and organic solvents and the presence of amino nitrogen, which allows soluble salts to be formed. These antibiotics as used in veterinary preparations are typically hydrochlorides, except for tiamulin, which is used as hydrogen fumarate. For the sake of simplicity, the names of the free antibiotics are used in this paper.

The similarity of physical and chemical properties hinders the determination of these compounds, particularly if they are present together. An additional problem is related to the presence of various auxiliary substances used in the preparation of veterinary drugs; apart from the active substances, these various additives can comprise ≥70% of the drug preparations. These auxiliary substances, often with similar physical and chemical properties, can hinder the determination of the antibiotic constituents. Therefore, methods for determining individual constituents are still needed.

Most published methods for the determination of oxytetracycline in various materials use liquid chromatography (LC; 1–5), polarography (6), thin-layer chromatography (TLC; 7, 8), spectrophotometry, and microbiological techniques (9). Similar methods are used for the determination of spectinomycin (10–15) and lincomycin (16–20).

The antibiotics mentioned above, except tiamulin, have been included in various pharmacopoeias (21–23) in which quantitative analysis is based on microbiological, spectrophotometric, gas chromatographic (GC), and LC methods, depending on the particular drug and analytical requirements. The method for the determination of tiamulin by GC with electron-capture detection after hydrolysis and derivatization (24) appears interesting.

In this paper, the conditions established for TLC identification and densitometric determination of oxytetracycline, lincomycin, spectinomycin, and tiamulin in veterinary drugs are presented.

**Experimental**

**Apparatus**

(a) **Densitometer.**—TLC Scanner 3 with CATS 4 software (Camag, Muttenz, Switzerland).

(b) **Sample applicator.**—Linomat IV (Camag).

(c) **Computer.**—PC Pentium MMX, with 16 MB RAM and Hewlett-Packard Laserjet 6L printer.

(d) **TLC plates.**—10 × 10 cm, cut from 20 × 20 cm aluminum TLC sheets precoated with silica gel (Cat. No. 1.05553, E. Merck, Darmstadt, Germany).

(e) **TLC chamber.**—20 × 10 × 5 cm (E. Merck, Cat. No. 1.11622).

**Reagents**

(a) **Standard solutions.**—Comparable substances were dissolved in methanol so that the following solutions were ob-
Table 1. Results for the identification of oxytetracycline, tiamulin, lincomycin, and spectinomycin in veterinary preparations

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Wavelength of maximum absorbance, nm</th>
<th>Spot color</th>
<th>Visualization reagent</th>
<th>Detection limit, µg</th>
<th>Rf</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytetracycline^a</td>
<td>350</td>
<td>Brown</td>
<td>16% H₂SO₄</td>
<td>0.07^b</td>
<td>0.51</td>
</tr>
<tr>
<td>Tiamulin^a</td>
<td>—</td>
<td>Yellow</td>
<td>16% H₂SO₄</td>
<td>0.02</td>
<td>0.08</td>
</tr>
<tr>
<td>Lincomycin^d</td>
<td>—</td>
<td>Brown</td>
<td>16% H₂SO₄</td>
<td>0.15</td>
<td>0.70</td>
</tr>
<tr>
<td>Spectinomycin^d</td>
<td>—</td>
<td>Yellow</td>
<td>Ehrlich’s reagent</td>
<td>0.42</td>
<td>0.29</td>
</tr>
</tbody>
</table>

^a Mobile phase used: 10% citric acid solution–n-hexane–ethanol (80 + 1 + 1, v/v).

^b At 350 nm.

^c At 430 nm.

for every solution. The final result was the mean value of the 3 measurements. The results are presented in Figure 3.

To evaluate the limits of determination, peak area measurements were taken into consideration, as they were in the investigation of detection limits; the limit of determination was the peak area for the relevant antibiotic that was 10 times larger than the other background peaks.

In further investigations, the recovery of each antibiotic was determined. For this purpose, 80–120% of the reference standards weighed with an accuracy of ±0.1 mg were added to weighed samples of the preparations, after the determination of each constituent, and the determination of each antibiotic was performed 5 times.

After the conditions of the quantitative analysis were optimized, each antibiotic in the preparations was determined with statistical analysis of the results.

Results and Discussion

The aim of this paper was to establish the optimum conditions for identification and simultaneous quantitation of oxytetracycline, tiamulin, lincomycin, and spectinomycin when present together in veterinary preparations. TLC/densitometry has been used for the analysis of mixtures containing substances from different chemical and pharmaceutical groups (25–27).

It was found that oxytetracycline showed a characteristic spectrum before and after visualization of the chromatogram with 16% sulfuric acid. Tiamulin and lincomycin gave spectra only after visualization of the chromatogram with sulfuric acid solution, whereas the spectrum of spectinomycin was obtained after reaction of the chromatogram with Ehrlich’s reagent.

In addition to the characteristic spectra obtained densitometrically, particular spots differed in color and contrasted with the white background of the stationary phase. The \( R_f \) values of the spots on the chromatograms were determined, and detection limits for the analytes were established.

It was found that both identification and quantitation of the drugs under consideration can be performed simultaneously, precisely, and rapidly under the established conditions.

Preliminary results have indicated that commercial TLC plates and 2 mobile phases, namely, \( n \)-butanol–ethanol–chloroform–25% ammonia \((4 + 5 + 2 + 5, \text{v/v})\) and 10% citric acid solution–\( n \)-hexane–ethanol \((80 + 1 + 1, \text{v/v})\), ensure good separation of the antibiotics present together in the preparations analyzed. In the case of oxytetracycline, in contrast with the other antibiotics, identification can be made directly under UV light, before derivatization, if the spectrum is recorded from 200 to 400 nm, or, as for the other antibiotics, after visualization of the chromatograms (with 16% sulfuric acid for oxytetracycline, tiamulin, and lincomycin, and with Ehrlich’s reagent for spectinomycin), if the spectrum is recorded from 200 to 800 nm (Figure 1).

It is quite obvious that only oxytetracycline can be determined from direct UV measurements, because the other compounds show no characteristic spectra within this range, as could be concluded from their chemical structures. Identification can also be made on the basis of spot location \((R_f)\) and appearance.

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Figure 1. Spectra recorded directly from chromatograms of constituents under analysis: (a) oxytetracycline before visualization; (b) oxytetracycline after visualization; (c) tiamulin; (d) lincomycin; and (e) spectinomycin. The investigation was performed for standard solutions under the conditions described in the text.
Figure 2. Densitogram and chromatogram for (1) tiamulin and (2) oxytetracycline, obtained by using 10% citric acid solution–n-hexane–ethanol (80 + 1 + 1, v/v) as the mobile phase, after visualization with 16% sulfuric acid.

Figure 3. Relationship of concentration vs peak area for (a) oxytetracycline (UV), (b) oxytetracycline (visible), (c) tiamulin, (d) lincomycin, and (e) spectinomycin. Every line is described by the equation $y = ax + b$, where $a = 2.660$ and $b = -0.259$ for (a), $a = 5.545$ and $b = -6.128$ for (b), $a = 1.788$ and $b = -0.024$ for (c), $a = 2.003$ and $b = 0.380$ for (d), and $a = 1.522$ and $b = 0.036$ for (e).
It should be noted that compact spots of uniform coloration and in sharp contrast with the background were obtained under the established conditions. Such features are of great importance in densitometric analysis. The results of the qualitative analysis are listed in Table 1.

Densitometric analysis led to symmetrical, well-developed peaks (Figure 2). Their areas varied linearly with concentration (Figure 3).

The results of the determination and the statistical analysis, which are presented in Table 2, indicate the repeatability and accuracy of the method. This is clearly visible from the results obtained for the individual antibiotics as well as from the statistical data. Individual results are consistent with the concentrations of active substance, as specified by the manufacturer.

The established limit of detection (Table 1) and limit of quantitation (Table 2) indicate the high sensitivity of the method in both qualitative and quantitative analyses. The recovery of all antibiotics determined under the established conditions was high, in the 100.01–102.54% range.

In conclusion, it should be stressed that the method presented in this paper enables us to perform simultaneous, quick, and precise identification as well as simultaneous determination of oxytetracycline, tiamulin, lincomycin, and spectinomycin when present together in veterinary preparations.

References

(23) *Farmakopea Polska* (1996) P.T. Farm., Warszawa, Poland

Table 2. Results for the determination of oxytetracycline, tiamulin, lincomycin, and spectinomycin in veterinary preparations

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Wavelength, nm</th>
<th>Declared weight, g</th>
<th>Recovery, %</th>
<th>Mean weight found ± SD, g&lt;sup&gt;a&lt;/sup&gt;</th>
<th>CV, %&lt;sup&gt;b&lt;/sup&gt;</th>
<th>CI&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Quantitation limit, µg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytetracycline</td>
<td>350</td>
<td>30.00</td>
<td>100.01</td>
<td>30.01 ± 0.1689</td>
<td>1.78</td>
<td>30.01 ± 0.38</td>
<td>0.28</td>
</tr>
<tr>
<td>Oxytetracycline</td>
<td>430</td>
<td>30.00</td>
<td>100.03</td>
<td>30.24 ± 0.3799</td>
<td>3.97</td>
<td>30.24 ± 0.86</td>
<td>0.63</td>
</tr>
<tr>
<td>Tiamulin</td>
<td>450</td>
<td>10.00</td>
<td>100.66</td>
<td>10.19 ± 0.1247</td>
<td>3.87</td>
<td>10.19 ± 0.28</td>
<td>0.04</td>
</tr>
<tr>
<td>Lincomycin</td>
<td>278</td>
<td>2.20</td>
<td>102.54</td>
<td>2.27 ± 0.0353</td>
<td>4.91</td>
<td>2.27 ± 0.08</td>
<td>0.20</td>
</tr>
<tr>
<td>Spectinomycin</td>
<td>421</td>
<td>2.20</td>
<td>101.55</td>
<td>2.18 ± 0.0324</td>
<td>4.69</td>
<td>2.18 ± 0.07</td>
<td>0.50</td>
</tr>
</tbody>
</table>

<sup>a</sup> n = 10; SD = standard deviation.
<sup>b</sup> CV = coefficient of variation.
<sup>c</sup> CI = confidence interval at 95% probability.