Quantitative Determination of Phospholipids in a Pharmaceutical Drug by Scanning and Video Densitometry

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This paper describes a convenient and practice method for quantitation of surfactant phospholipids (1,2-dipalmitoyl-3-sn-phosphatidyl choline [DPPC] and 1-palmitoyl-2-oleyl-3-sn-phosphatidyl glycerol [POPG]) in a recombinant surfactant lyophile (Venticute®) by high-performance, thin-layer chromatography (HPTLC) with video densitometry. DPPC and POPG were extracted from Venticute-lyophile using methanol. Separation from the other active ingredients and excipients was accomplished by HPTLC on silica gel F254 plates with a mixture of chloroform, methanol, glacial acetic acid, and water as development solvent. Postchromatographic derivatization by dipping in copper sulphate/phosphoric acid reagent and subsequent heating shows grey-brown bands on a light blue background. These were detected with the video densitometer in the VIS range, and with scanning densitometry at 365 nm. Linear calibration in a working range of 0.7–1.3 \( \mu \text{g} \) DPPC and 0.35–0.65 \( \mu \text{g} \) POPG was demonstrated by integrating the area under the peaks. Good results were obtained with recovery experiments. When compared to classical slit scanning densitometry, video densitometry represents a fast alternative to quantitate thin-layer chromatograms in surfactant phospholipid analysis.

Venticute® is the first human homologous recombinant surfactant for the treatment of Acute Respiratory Distress Syndrome (ARDS). It consists of recombinant surfactant protein rSP-C embedded in a phospholipid matrix (rSP-C Surfactant). The phospholipid matrix is formed by 70% 1,2-dipalmitoyl-3-sn-phosphatidyl choline (DPPC) and 30% 1-palmitoyl-2-oleyl-3-sn-phosphatidyl glycerol (POPG), together with a small amount of palmitic acid.

Although liquid chromatography (LC) has been widely promoted for phospholipid analysis in the last years, several features of thin-layer chromatography (TLC) and high-performance thin-layer chromatography (HPTLC) show that these techniques are still particularly useful for this application (1–3). A benchmark study showed a considerable cost reduction without loss in accuracy and precision when defined phospholipids were analyzed using HPTLC with scanning densitometry as compared with HPLC with light-scattering detection (4).

As DPPC and POPG are considered to be active ingredients not excipients of the rSP-C Surfactant Venticute, they have to be assayed in the finished product. This is actually done using HPTLC with scanning densitometry. Video densitometry was considered a fast and convenient alternative to scanning densitometry that would combine both quantitation and documentation. The known disadvantages of video densitometry—lower sensitivity and precision, especially when analyzing low concentrations (5)—do not negatively influence this application, because the phospholipids are the main components of the analyte and specification limits are set to 90–110% of the label claim.

This paper describes our method for quantitative determination of DPPC and POPG by scanning and video densitometry.

Experimental

Apparatus

(a) UV Scanner.—Scanning densitometry was performed with a Desaga (Wiesloch, Germany) UV scanner in absorbance/reflectance mode at a wavelength of 365 nm (measured with slit dimensions of 1.0 × 3.0 mm) and processed with the cd60 software (Desaga).

(b) Video.—For video densitometry the Camag Video Documentation system VideoStore 2 (Camag, Muttenz, Switzerland) was used. The system includes a UV/VIS lamp and a Hitachi 3-CCD color video camera HV-C20 with a horizontal resolution of 580 TV-lines and a standard sensitivity of 5.5 lx. An acquisition device digitizes images to 480 × 374 pixels with 256 grey shades. The plates are illuminated with white light. Quantitative evaluation of the plates was processed with the corresponding software VideoStore 1.01 and Video Scan 1.01 (Camag).

Reagents and Chemicals

Precoated HPTLC plates (20 × 10 cm) silica gel F254 (Merck, Darmstadt, Germany) were used for chromatography. All plates were precleaned with methanol and then air dried.
All other reagents and solvents were analytical grade. DPPC and POPG reference standards of defined purity were supplied by Byk Gulden (Konstanz, Germany).

**Postchromatographic Derivatization**

After development, the plates were air dried and then heated to 110°C for 7 min. After cooling, the plates were dipped into the dipping solution for 2 s up to the solvent front. The dipping solution consisted of 10% copper(II)-sulfate-5-hydrate solution (dissolved in 4% aqueous orthophosphoric acid). Immediately after dipping, the plate was dried in horizontal position and was then heated for 30 min at 170°C. DPPC and POPG appear as grey-brown spots on a light blue background.

**Sample Preparation**

Reference standard substances were dissolved in methanol to concentrations of 0.35–0.65 mg/mL DPPC and 0.15–0.35 mg/mL POPG. Venticute-lyophile (210 mg) was dissolved with 10 mL chloroform in a 50 mL flask, centrifuged (for ca 5 min), and filled up to 50 mL with chloroform.

<table>
<thead>
<tr>
<th>Method</th>
<th>Detection limit, ng</th>
<th>Quantitation limit, ng</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>POPG</td>
<td>DPPC</td>
</tr>
<tr>
<td>Scanner</td>
<td>22.3</td>
<td>18.1</td>
</tr>
<tr>
<td>Video</td>
<td>38.6</td>
<td>22.3</td>
</tr>
</tbody>
</table>

The clear supernatant was diluted to a concentration of ca 0.52 mg/mL DPPC and 0.22 mg/mL POPG.

**Quantitative Analysis**

Basic calibration was done by applying the 7 methanolic standard solutions of the substances in amounts of 2 µL to the plates with a Hamilton 100 µL syringe using a Linomat IV sample applicator (Camag) with a spraying speed of 8 s/µL. For routine analysis, a 3-point calibration was used, applying 2 µL of the sample and 2 µL of 3 standard concentrations of 0.4, 0.5, and 0.6 µg/µL DPPC and 0.2, 0.25, and 0.3 µg/µL POPG. The solutions were applied as 5 mm bands starting at 50 mm, 6 mm apart, and 15 mm from the bottom of the TLC plate. The HPTLC plates were developed over a migration distance of 7 cm with a mixture of chloroform, methanol, glacial acetic acid, and water (28 + 8 + 3.2 + 2, v/v) in a filter paper-lined twin-trough chamber (previously saturated for 5 min) with developing solvent.

![Sample (line) and standard (dots) chromatogram of DPPC and POPG.](image-url)
Results and Discussion

Comparison of the 2 evaluation techniques showed good agreement of the results. The active substances POPG and DPPC were well separated (Figure 1) and detectable after postchromatographic derivatization as sharp spots (Figure 2). Calibration data were fitted with linear regression curves which are verified by Mandels linearity test (Figures 3 and 4). Excellent correlation coefficients of 0.998 for scanner and video system (0.998 for DPPC and 0.989 for POPG) were obtained in the calibration range of 50–350 ng for DPPC and 25–350 ng for POPG. Quantitation and detection limits were

![Figure 2. HPTLC plate after dipping and heating (S1...S3 = standard concentration 1...3, LSF = sample of Venticute<sup>®</sup>).](image)

![Figure 3. Linear calibration curve of POPG obtained by video densitometry (α = 0.05; R = 0.9967).](image)
determined by the linear calibration functions of DPPC and POPG. Whereas the scanner shows lower detection and quantitation limits, the imaging system (Table 1) offers faster analysis time and the possibility of full documentation by storage of the actual image of the plate. The recovery experiments show comparative high standard deviations of 6–7% for both systems. Higher deviations might be attributed to the postchromatographic derivatization. Recovery experiments were performed by adding DPPC and POPG to a mixture of excipients and other active substances ready for use. The recovery was always higher than 100% with the scanner as well as with the image analyzer (Table 2). Considering the known problems with HPLC using light-scattering detection and the wide specification limit, the above results prove the excellent applicability of video densitometry for quantitative routine analysis of phospholipids.

Acknowledgment

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References