A simple, sensitive, and reproducible high-performance thin-layer chromatographic method using densitometry is presented for the determination of naproxen in rat serum. Only 0.1 mL serum was used for extraction. Separations were performed on 10 x 10 cm plates coated with silica gel, with toluene-ethyl acetate-acetic acid (82 + 15 + 3) as the mobile phase. Benzophenone was used as the internal standard. Quantification was performed by densitometry at 260 nm. The response for naproxen was linear (r = 0.992) over the range 2–100 mg/L. Method validation demonstrated good recoveries (92–96%), sensitivity (limit of quantitation, 1 mg/L), repeatability of sample application (4%), repeatability of the method (8%), and intermediate precision (5%). The procedure was applied to the quantitation of naproxen in rat serum.

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Naproxen, (S)-6-methoxy-α-methyl-2-naphthaleneacetic acid, is used in painful osteoarthritis and rheumatoid arthritis. Activity resides mainly in the S-enantiomer. Naproxen inhibits cyclooxygenase and decreases prostaglandin concentrations in fluids and tissues. Some side effects have been described, such as gastrointestinal bleeding and reduction of kidney function (1). Therapeutic activity based on clinical indexes is established with a daily dose of 500–1500 mg (2). Naproxen is primarily metabolized to 6-O-demethylnaproxen, and both compounds are conjugated to their glucuronides.

To determine the plasma concentration of naproxen, a number of analytical techniques are described in the literature including spectrofluorimetry (3–5), gas chromatography (6–14), and recently spectrophotometry (15). Actually, liquid chromatographic (LC) methods seem to be more convenient. A wide variety of reversed-phase LC methods with various sensitivities have been reported for the analysis of small plasma samples (16–21). Recently, electrokinetic capillary chromatography was also used successfully (22). These procedures used sample pretreatments such as solvent extraction, protein precipitation, or solid-phase extraction. Thin-layer chromatography (TLC) was used less frequently (23–25), and the methods described in these references combined TLC with spectrofluorimetry. They are tedious and time consuming and require external standardization. High-performance (HP) TLC was not applied to the determination of naproxen in plasma; however, it was used for the determination of ibuprofen (26) and flurbiprofen (27).

In this study, we describe a simple, rapid, sensitive, and specific HPTLC method for the determination of naproxen in serum. Compared with the LC methods, the HPTLC assay is more attractive because of its speed and simplicity. HPTLC facilitates quantitation by scanning and analysis of several samples simultaneously. The proposed method features naproxen extraction with a small serum volume and densitometric quantitation using an internal standard.

Experimental

Reagents

All solvents (spectroscopic grade) used were from Merck (Manheim, Germany). Naproxen and benzophenone, which were used as the internal standard, were from Sigma (St. Louis, MO).

Apparatus

Chromatography was performed on 10 x 10 cm HPTLC plates precoated with Silica Gel 60, 200 μm layer thickness (Merck, Darmstadt, Germany). Samples were applied to the plate with a Camag (Muttenz, Switzerland) microapplicator, 8 mm from the lower edge of the plate and 10 mm apart, starting 10 mm from the side of the plate. Development was performed in a linear developing chamber (Camag). Zones were quantitated by densitometry with a TLC/HPTLC Scanner III and CATS 4 software (Camag) for a distance of 8 cm at ambient temperature by using a measurement wavelength of 260 nm and slit dimensions of 5 x 0.1 mm. Each plate was scanned 3 times. Peak areas were used for quantitation. Average quantitative results were given. Scanning speed was kept...
at 1 mm/s. Plates were developed with several mobile phases containing toluene, ethyl acetate, and acetic acid.

**Sample Preparation**

Serum samples were collected from Wistar rats that received naproxen intraperitonially at 8 mg/kg. Blood samples were collected from the orbital venous plexus. Serum samples were separated by centrifugation at 6000 rpm for 15 min. To improve sample stability, they were stored at −20°C and allowed to defrost at 25°C before use. Each serum sample was analyzed 10 times.

To 0.1 mL serum were added 0.01 mL of a 500 mg/L solution of benzophenone (internal standard) in methanol and 0.02 mL 1M HCl. Extraction was performed with 2 mL diethyl ether. The organic layer was transferred to a clean test tube and evaporated to dryness. The dry residue was reconstituted with 0.1 mL mobile phase, and 1 μL was spotted on the plate. Spotting >1 μL on the plate saturates the silica gel and introduces tailing that affects peak area and resolution.

**Calibration**

Calibration was accomplished by using the following procedure. To ten 0.08 mL samples of blank serum were added 0.01 mL of various concentrations of naproxen (20–1000 mg/L) and 0.01 mL of a constant internal standard concentration (500 mg/L). In the samples obtained, the naproxen concentration varied from 2 to 100 mg/L, and the internal standard concentration was fixed at 50 mg/L. The samples were extracted and analyzed according to the procedure described above to establish the calibration curve in the presence of serum. The use of an internal standard limits the errors occurring during the naproxen extraction. The advantages of this calibration method are that the naproxen quantities injected need not be accurately measured because the detector responses do not alter the area ratio.

The calibration curve in the absence of serum was established by analyzing standard solutions in which the naproxen concentration varied from 2 to 100 mg/L, and the internal standard concentration was fixed at 50 mg/L. The solutions were obtained as follows. To ten 0.08 mL portions of methanol were added 0.01 mL of various concentrations of naproxen (20–1000 mg/L) and 0.01 mL of a constant internal standard concentration (500 mg/L). In the solutions obtained, the naproxen concentration varied from 2 to 100 mg/L, and the internal standard concentration was fixed at 50 mg/L. The solutions were analyzed according to the procedure described above to establish the calibration curve in the absence of serum.

**Detection Limit**

The detection limit was determined at a signal-to-noise ratio of 4:1.

**Recovery**

At a specific concentration, recovery was determined from the ratio of the calibration curves in the presence and in the absence of serum.

**Versatile Application**

The within-run precision of sample applications was estimated from 10 analyses of the same aliquot of blank serum containing added naproxen and internal standard. To 0.08 mL serum were added 0.01 mL of a constant concentration of naproxen (500 mg/L) and 0.01 mL internal standard in methanol at 500 mg/L. The sample was extracted and spotted 10 times on the same plate.

**Repeatability**

Repeatability was determined by analyzing ten 0.8 mL portions of blank serum containing added naproxen and internal standard. To each 0.8 mL blank serum was added 0.1 mL of a constant concentration of naproxen (500 mg/L) and 0.1 mL internal standard at 500 mg/L. The 10 samples were extracted and spotted on the same plate.

**Results and Discussion**

**Mobile Phase Selection**

HPTLC experiments were conducted with mobile phases containing ethyl acetate, toluene, and acetic acid in which the amounts of toluene and ethyl acetate were changed. The variation of the retention factors of the internal standard and naproxen with the ethyl acetate content of the mobile phase is shown in Figure 1. Below 10% ethyl acetate, the retention factor, \( R_F \), of naproxen is too low; its corresponding peak could interfere with the endogenous compounds present in serum and appearing with a small \( R_F \). Above 30% ethyl acetate, the \( R_F \) of the internal standard is near one. The choice of the best chromatographic conditions was a compromise that depended on the retention factors of naproxen and benzophenone. It appeared that the best separation between naproxen and the internal standard occurred with toluene–ethyl acetate–acetic acid (82 + 15 + 3). The retention factors of naproxen and benzophenone obtained with this mobile phase were 0.31 and 0.73, respectively. Retention factors obtained for some other anti-inflammatory drugs with the proposed HPTLC method.
were 0.10 for flurbiprofen, 0.17 for ibuprofen, 0.40 for indoprofen, and 0.47 for ketoprofen. Naproxen was clearly separated from the other drugs. We did not study the possible interferences of naproxen metabolites because in plasma only naproxen could be detected (20). The proposed mobile phase is different from the pharmacopeial mobile phase (28), toluene–tetrahydrofuran–acetic acid (30 + 3 + 1), which is more suited to the determination of the degradation products in raw material.

**HPTLC Results**

The therapeutic range of naproxen in serum is between 5 and 50 mg/L. The calibration graph was obtained for the range 2–100 mg/L. The least-squares regression equation of the calibration curve was linear. Statistical treatment of the calibration curves in the presence or absence of serum was estimated through the slope and intercept relative errors. Data for the calibration curve in the absence of serum were as follows: slope, $42 \times 10^{-4}$; relative error of slope, 2%; intercept, $9.6 \times 10^{-4}$; relative error of intercept, 6%; and correlation coefficient, 0.996. Data for the calibration curve in the presence of serum were as follows: slope, $44.7 \times 10^{-4}$; relative error of slope, 4%; intercept, $11.7 \times 10^{-4}$; relative error of intercept, 8%; and correlation coefficient, 0.992.

The detection limit of the assay (determined with a baseline signal-to-noise ratio of 4:1) in the proposed method is 1 mg/L. A lower detection limit can be found at a lower UV wavelength (for example, at 230 nm), but endogeneous interferences in the naproxen determination may occur. Indeed, at a retention factor of 0.31, which corresponds to that of naproxen, the 230 nm baseline absorbance of the blank serum (labeled naproxen 230 nm in Figure 2b) extract is high when compared with the corresponding 260 nm baseline absorbance (labeled naproxen 260 nm in Figure 2b).

Recovery was determined from the ratio of the calibration curves in the presence and the absence of serum. The values found were satisfactory over the entire therapeutic range. For the lowest naproxen concentrations (1–5 mg/L), recoveries were 92–94%; for naproxen concentrations of 5–50 mg/L, recoveries were 94–96%.

The other results, determined as described in the Experimental section, reveal the good repeatability of sample application (4%), repeatability of the method (8%), and intermediate precision (5%).

**Table 1. Determination of naproxen in rat serum by the proposed HPTLC method**

<table>
<thead>
<tr>
<th>Rat</th>
<th>Time of serum collection, h after dosing</th>
<th>Naproxen found, mg/L</th>
<th>Relative standard deviation, %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.5</td>
<td>70</td>
<td>2.9</td>
</tr>
<tr>
<td>2</td>
<td>2</td>
<td>81</td>
<td>3.1</td>
</tr>
<tr>
<td>3</td>
<td>2</td>
<td>83</td>
<td>3.6</td>
</tr>
<tr>
<td>4</td>
<td>2</td>
<td>77</td>
<td>3.6</td>
</tr>
</tbody>
</table>

* Each value is the average of 10 determinations.
Rat serum samples were analyzed by the proposed HPTLC method. A typical chromatogram is shown in Figure 2c. No interfering peaks were noticeable (see chromatogram of blank serum in Figure 2a). The quantitative results are given in Table 1.

Conclusions

The HPTLC method enables quantitation of naproxen in rat serum in the concentration range of 2–100 mg/L. It includes solvent extraction after addition of an internal standard, evaporation to dryness, residue dissolution, and densitometric measurement after chromatographic separation. The repeatability of sample application, the repeatability of the method, the intermediate precision, the linearity, and the recoveries by the proposed method are excellent. Only 0.1 mL serum is needed for analysis. The limit of detection was found to be 1 mg/L when the therapeutic range of naproxen in serum was between 5 and 50 mg/L. The assay described is attractive because of its speed, simplicity of sample treatment, and the large number of samples that can be analyzed simultaneously with a small quantity of solvent. It can be used for routine analysis in the clinical laboratory after its adaptation to samples of human serum.

References