Liquid-Chromatographic Quantification Compared with Gas-Chromatographic–Mass-Spectrometric Determination of Verapamil and Norverapamil in Plasma

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A high-performance liquid chromatographic (HPLC) method for determining verapamil and norverapamil in plasma is presented and compared with gas chromatography/mass spectrometry (GC-MS). The plasma samples were extracted at alkaline pH with hexane containing 2-butanol (20 mL/L) and then back-extracted into phosphate buffer (0.1 mol/L, pH 3.0). For chromatography we used a reversed-phase column (Supelcosil LC-18 DB) with a mobile phase of the phosphate buffer and acetonitrile (70/30 by vol). Fluorescence detection was used (excitation at 203 nm, emission at 320 nm). Overall analytical recovery was 85%. Standard curves were linear from 1 to 1000 µg/L. The detection limit was 1 µg/L. The assays are accurate and precise. We found no interferences by those substances tested. Results by HPLC and GC-MS agreed well (r = 0.99) for both verapamil and norverapamil determinations.

Additional Keyphrases: antiarrhythmic drugs • antihypertensive drugs • chromatography, reversed-phase

Verapamil, the prototype of calcium channel blocking agents, has been used for more than 20 years for treating angina pectoris and supraventricular tachyarrhythmias, and it is also an effective antihypertensive agent.

Verapamil has been assayed by spectrofluorometry (1), gas chromatography (2–5), liquid chromatography (6), high-performance liquid chromatography (HPLC) (7–8), and gas chromatography/mass spectrometry (GC-MS) (9–10).

HPLC has been the technique most frequently used in analysis for verapamil and norverapamil in biological materials, owing to the technique’s relative simplicity and low cost. However, to our knowledge, there are no reports comparing HPLC with GC-MS. Therefore, we compared a newly developed HPLC method with our previous GC-MS method (11) for determination of verapamil and norverapamil in human plasma.

Materials and Methods

Material. Verapamil HCl, norverapamil HCl, the internal standards (D517, verapamil analog, [3H]verapamil, and [3H]norverapamil) were from Knoll AG, Ludwigshafen, F.R.G. Acetonitrile, hexane, and 2-butanol were of "HPLC" grade (Fison, Loughborough, U.K.). The drugs tested for interference were from the Karolinska Pharmacy. All other chemicals were of analytical grade (Merck, Darmstadt, F.R.G.).

Sample preparation. We prepared a standard concentrations curve, used for both HPLC and GC-MS, by adding known amounts (1–1000 µg/L) of verapamil and norverapamil to drug-free human plasma. These standards were then divided into 1-mL aliquots and stored at −20 °C.

We added internal standards to patients’ samples and analyzed them by both HPLC and GC-MS.

HPLC analysis. To 0.5 mL of plasma add 100 µL of the internal standard solution (D517, 1000 µg/L in water), 0.5 mL of a 1 mol/L solution of sodium hydroxide, and 2.5 mL of hexane containing 2-butanol (20 mL/L). Extract vigorously for 1 min, then centrifuge (1000 × g, 10 min). Aspirate the organic phase and transfer it to a tube containing 0.1 mL of phosphate buffer (0.1 mol/L, pH 3.0). After extraction and centrifugation, aspirate and discard the organic phase. From the remaining acidic phase, inject 50 µL into the liquid chromatograph.

We used an HPLC system consisting of a Constametric III pump (LDC, Riviera Beach, FL), a sample injection valve (Model 7125; Rheodyne Inc., Cotati, CA) fitted with a 50-µL sample loop, a Model 970 FS fluorescence detector (Schoeffel, Westwood, NJ), and a dual-pen recorder (Servogor, Vienna, Austria). For chromatographic separation we used a 150 × 4.0 mm column of Supelcosil LC-18 DB (particle size 5 µm; Supelco, Bellafonte, PA). The mobile phase consisted of a 0.1 mol/L solution of orthophosphoric acid (adjusted to pH 2.0 with triethylamine, then adjusted to pH 3.0 with KOH, 5 mol/L) and acetonitrile (70/30 by vol). The flow rate was 1.5 mL/min. The detector was set at an excitation wavelength of 203 nm and an emission wavelength of 320 nm with a cutoff filter.

GC-MS analysis. For comparison, we used a GC-MS method described previously (10, 11). To 1.0 mL of plasma add 100 µL of internal standard solution (500 µg of [3H]verapamil and 2000 µg of [3H]norverapamil per liter of water), 1.0 mL of 1 mol/L sodium hydroxide, and 5.0 mL of toluene/2-butanol (9/1 by vol). Shake the mixture for 15 min and centrifuge at 500 × g for 10 min. Transfer the organic phase to a tube containing 1.0 mL of a 50 mmol/L solution of sulfuric acid. Shake the mixture again for 15 min and centrifuge as before. Discard the organic phase and add 4.0 mL of a 5 mol/L solution of ethyl chlorofomate in dichloromethane and 1.0 mL of saturated carbonate buffer (pH 9.1). Shake the mixture for 10 min, then centrifuge. Transfer the organic phase to another tube and remove the solvent at 50 °C under a stream of nitrogen. Dissolve the residue in 50 µL of chloroform and use 2 µL for analysis by GC-MS with selected ion monitoring.

For GC-MS we used a Model 4500 gas chromatograph–mass spectrometer (Finnigan Corp., Sunnyvale, CA), equipped with the INCOS data system, and a 12 m × 0.32 mm fused-silica capillary column coated with SE-30 (Orion, Separation Research, Abo, Finland). Helium was used as carrier-gas at a flow rate of 1.5 mL/min. The temperature of the column was 270 °C and the samples were introduced by the falling-needle technique. The ion source was operated at 280 °C with an ionization energy of 50 eV and an emission current of 0.3 mA. The mass spectrometer was adjusted to record the ions m/z 303 and m/z 306 for verapamil and its deuterated internal standard and m/z 361 and m/z 364 for norverapamil and its deuterated internal standard.

The relative standard deviation (CV, %) of the method at 10 µg/L was 2.7% for verapamil and 4.7% for norverapamil (n = 10 each).
Results and Discussion

Chromatography. Verapamil, norverapamil, and the internal standard were well resolved, with sharp, symmetrical peaks, on the column we used, Supelcosil LC-18 DB, deactivated for basic compounds (Figure 1). Adding triethylamine to the mobile phase shortened the retention times without affecting the separation. The time needed for separation of verapamil and norverapamil (8 min) is shorter than that earlier reported for a reversed-phase system (8).

Sample preparation. The use of hexane containing 2-butanol (20 mL/L) in the first extraction step avoids emulsions and results in a good analytical recovery for both verapamil and norverapamil. Back-extraction into an acidic water phase is necessary to eliminate a substance represented by a peak that interferes with the analysis of verapamil. Overall analytical recovery of verapamil and norverapamil added to plasma was 85%.

In other methods either heptane (3) or diethyl ether (7, 8) was used to extract verapamil and norverapamil. However, heptane extracts a low proportion (5–20%) of verapamil (3), and use of diethyl ether requires large volumes, to avoid emulsion formation (7, 8).

Standard curves and detection limits. The plasma standard curves for verapamil and norverapamil are both linear in the range 1–1000 μg/L (r = 0.99). The limits of detection for both compounds in plasma are 1 μg/L.

Precision. We analyzed 10 samples containing 5 ng of verapamil and 5 ng of norverapamil per milliliter and 10 samples containing 100 ng each of verapamil and norverapamil per milliliter. Precision was good for both verapamil and norverapamil at the concentration tested (Table 1).

Comparison of HPLC and GC-MS. Using both methods, we analyzed plasma samples from patients who were receiving verapamil orally. The concentrations of verapamil in plasma ranged between 3 and 856 μg/L, those of norverapamil between 3 and 445 μg/L. Regression analysis of results by the GC-MS (y) and HPLC methods (x) showed a statistically significant correlation between the two: verapamil: y = 0.99x – 0.81 μg/L (P < 0.001); norverapamil: y = 0.98x – 1.04 μg/L (P < 0.001).

We conclude that use of HPLC with fluorescence detection is a good alternative to GC-MS for analyzing for verapamil and norverapamil in patients' samples obtained in clinical practice. The HPLC method described here involves a rapid extraction with solvent and gives a high analytical recovery of verapamil and norverapamil from plasma. The short retention times for the substances investigated are an additional advantage.

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Table 1. Precision of the HPLC Method for Verapamil (V) and Norverapamil (NV)

<table>
<thead>
<tr>
<th>Added</th>
<th>Found, mean (SD) V</th>
<th>Found, mean (SD) NV</th>
<th>CV, %</th>
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</thead>
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<tr>
<td>5</td>
<td>5.1 (0.1)</td>
<td>5.4 (0.1)</td>
<td>2.5</td>
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<tr>
<td>100</td>
<td>101.9 (2.6)</td>
<td>101.6 (2.7)</td>
<td>2.5</td>
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</tbody>
</table>

n = 10 each.

Fig. 1. Chromatograms of (A) blank plasma containing the internal standard (scale factor 0.04 μA/peptide) and (B) plasma sample from a patient treated with verapamil.

Scale factor 0.2 μA. The peaks correspond to 46 ng of verapamil and 78 ng of norverapamil per milliliter. IS, internal standard; NV, norverapamil; V, verapamil.

References