Quantitation of Verapamil and Norverapamil in Small Blood Samples From the Rat by High Performance Liquid Chromatography

S. A. Hamadi a, S. L. Chang a, P. J. Welund a

a Colleges of Pharmacy and Agriculture University of Kentucky Lexington, Kentucky

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QUANTITATION OF VERAPAMIL AND NORVERAPAMIL IN SMALL BLOOD SAMPLES FROM THE RAT BY HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

S.A. Hamadi, S.-L. Chang and P.J. Wedlund
Colleges of Pharmacy and Agriculture
University of Kentucky
Lexington, Kentucky 40536

Key words: Verapamil; Norverapamil; Rat; Blood; Samples; HPLC

ABSTRACT

A simple, sensitive HPLC assay using fluorescence detection was developed for quantitation of verapamil and its active metabolite, norverapamil in 100-200 μl blood samples from the rat. Baseline separation of verapamil, norverapamil and internal standard, propranolol, was attained within 14 minutes. Standard curves for verapamil and norverapamil were linear from 7 ng/ml to 1000 ng/ml with a limit of detection of 4 ng/ml for both compounds. The intraday and interday coefficients of variation in verapamil and norverapamil concentrations, determined from spiked whole blood samples, were less than 10%.

Send correspondence to: Dr. Peter J. Wedlund
College of Pharmacy
University of Kentucky
Lexington, KY 40536-0082
The development of sensitive and selective assay for verapamil and norverapamil in small blood samples was necessary in order to fully characterize their disposition in individual rats where blood volume is limited. Preliminary results following intravenous and oral dosing of verapamil in rat showed a rapid elimination and extensive first-pass removal of the drug in this species. Similar to observations in man, measurable norverapamil blood levels were found only after oral verapamil administration.

INTRODUCTION

Verapamil is a racemic cardiovascular drug which is used clinically to control supraventricular tachycardia.\(^1\) Consequently, a number of assays have been developed for verapamil and applied to investigations concerned with its disposition following administration to man.\(^2\)\(\text{-}^9\) To date, little information is available on verapamil disposition in rat, or the suitability of the rat as an animal model for studies examining factors which may influence verapamil systemic availability. For such studies, a sensitive assay for verapamil is required to detect low levels of this drug and its active metabolite in small biological samples.

The spectrophotometric assay originally used for quantitation of verapamil levels is neither selective nor sensitive enough for this work.\(^2\) The present GLC methods, which
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require at least 1 ml of plasma or blood and involve long extraction and/or derivatization procedures, are equally unsuitable for studies in rat. Although GC/MS is selective and sensitive, the accessibility to this equipment precludes its more general application. Several HPLC methods have been reported and appear to provide adequate sensitivity, but all employ an internal standard (D-517) which is not commercially available, and require significant amounts of blood or plasma (0.5 ml or more) for detection of lower verapamil levels. Two additional shortcomings of some HPLC methods include: 1) linearity over a limited concentration range; and 2) an inability to quantitate the concentration of any metabolite of verapamil. To overcome some of these limitations, a sensitive HPLC assay for quantitation of verapamil and norverapamil in small blood samples was developed. This assay allowed the disposition of verapamil to be characterized in the rat following its administration by the intravenous and oral routes.

EXPERIMENTAL

Materials and Reagents: Verapamil hydrochloride, norverapamil hydrochloride, propranolol hydrochloride, triethylamine, acetic acid and sodium hydroxide were used without additional purification. Methanol and acetonitrile were HPLC grade.

Instrumentation and Analytical Conditions: The HPLC system was equipped with a fluorescence detector employing an...
excitation wavelength of 203 nm and an emission cut off filter of 320 nm for all quantitation. The injector was equipped with a 50 µl sample loop. The guard column (4.1 mm x 5 cm, 35 µm C18 packing) and the analytical column (4.6 mm x 25 cm, 5 µm ultraspere ODS, Altex) were maintained at ambient temperature during analysis. Separation of analytes was achieved using a ternary mobile phase consisting of buffer:methanol:acetonitrile (42:24.5:33.5, v/v/v). The buffer portion of the mobile phase contained triethylamine (0.02 M) and acetic acid (0.02 M) and was buffered to pH 5.2 by the addition of glacial acetic acid. The flow rate for this system was 1.0 ml/min.

Standards: A stock solution containing 10.5 µg/ml each of verapamil and norverapamil was prepared in double distilled water. Serial dilutions of this stock solution were carried out to obtain concentrations of verapamil and norverapamil ranging from 7 ng/ml to 1000 ng/ml. A stock solution of propranolol containing 56 ng/ml was prepared for use as an internal standard.

Assay: Standard curves were prepared by adding 100 µl of whole blood to 100 µl of standard placed in a 10 ml PTFE-lined screw capped tube. For standards containing less than 25 ng/ml, 200 µl of whole blood and 200 µl of the corresponding standard were used. The blood was then mixed with 100 µl of internal standard, 50 µl of 1N NaOH and 5 ml of ethyl ether before centrifugation. The aqueous layer was then frozen in a dry ice-acetone bath and the ether layer transferred to a 5 ml
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Reacti-vial®. After evaporation of a portion of the ether layer under a stream of nitrogen, the ether was acidified with 100 μl of 0.25 N H₂SO₄. This mixture was vortexed for 1 minute and centrifuged before injecting 50 μl of the acidic aqueous phase into the HPLC. Blood samples were extracted similarly following the addition of 100 μl of internal standard.

Animal Study: Two Sprague Dawley rats weighing 250 gm were used to demonstrate the application of this assay to small biological samples. Each animal was anesthetized with pentobarbital (50 mg/kg) before cannulation of the right femoral vein and left femoral artery for blood sampling and drug dosing. Anesthesia was maintained subsequent to these cannulations for administration of verapamil and blood sample collection. One rat received 0.5 mg of verapamil dissolved in 0.1 ml of sterile saline by intravenous injection. The other rat was administered 2.5 mg of verapamil in 0.5 ml of saline by gastric intubation. Blood samples (220 μl) were collected from both rats via the femoral artery at 0, 2, 5, 10, 20, 30, 45, 60, 90, 120, 150, 180 and 240 minutes after drug administration using a microsampling technique.¹⁶ Blood samples collected from 0 to 90 minutes were assayed in duplicate (100 μl) for both verapamil and norverapamil. Blood samples collected after 90 minutes were assayed using 200 μl of whole blood. All blood samples were pipetted into 10 ml PTFE-lined screw capped tubes immediately after collection and were frozen until assayed.¹⁶
FIG. 1 Chromatograms of a blank blood sample (A) and a blood sample spiked with internal standard, norverapamil and verapamil (B). Retention times for the internal standard (IS), norverapamil (N) and verapamil (V) are 7.2, 11.2 and 12.8 minutes, respectively.
FIG. 2 Standard curves for verapamil (●) and norverapamil (○). The slope and correlation coefficient for the verapamil standard curve are 0.0073 and 0.999, respectively. The slope and correlation coefficient for the norverapamil standard curve are 0.0083 and 0.999, respectively.

RESULTS AND DISCUSSION

The ternary mobile phase for this assay was developed based on published optimization methods for liquid chromatography.\textsuperscript{17-20} The triethylamine used in the mobile phase of this assay has previously been reported to improve peak symmetry of basic drugs\textsuperscript{21-22} and was found to be a necessary component for optimum peak resolution. Representative chromatograms of a blank blood sample (A) and a blood sample spiked with verapamil, norverapamil
and internal standard (B) are shown in Fig. 1. The retention times for the internal standard, norverapamil and verapamil are 7.2, 11.2 and 12.8 minutes, respectively. All peaks were separated with baseline resolution, and no interfering substance was noticed in any blank blood samples.

Standard curves for verapamil and norverapamil were linear over a wide concentration range (7 ng/ml-1000 ng/ml) (Fig. 2) and the intercept for all standard curves was not significantly different from zero. The average slope for the verapamil and norverapamil standard curves were 0.0085 and 0.0088, respectively with coefficients of variation less than 7% for both slopes over a 2 month time period. The mean correlation coefficient (R) for ten separate verapamil and norverapamil standard curves over this same time period was 0.998 for each. The minimum detectable level of verapamil and norverapamil, set as a signal to noise ratio of 2, was 4 ng/ml when using 200 μl of whole blood.

The intraday and interday reproducibility of this assay was determined by spiking blood with verapamil and norverapamil in amounts indicated in Table 1. The intraday coefficient of variation in verapamil and norverapamil was then determined by assaying nine spiked blood samples of each concentration in one day. The results in Table 1 indicate a 4-5% intraday variation for verapamil and a 5-8% intraday variation in norverapamil concentrations. This variation was only slightly greater for samples assayed on separate days (Table II). The interday
TABLE I
Intraday Assay Variability of Verapamil and Norverapamil in Blood
(Nine determinations for each concentration)

<table>
<thead>
<tr>
<th>Verapamil Conc. (ng/ml)</th>
<th>Coefficient of Variation (%)</th>
<th>Norverapamil Conc. (ng/ml)</th>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.6</td>
<td>4.46</td>
<td>23.8</td>
<td>4.85</td>
</tr>
<tr>
<td>151.5</td>
<td>4.38</td>
<td>159.0</td>
<td>5.85</td>
</tr>
<tr>
<td>787.5</td>
<td>5.03</td>
<td>795.0</td>
<td>8.21</td>
</tr>
</tbody>
</table>

variability in verapamil levels ranging from 3-7% and 4-9% for norverapamil.

The blood concentrations of verapamil over time following a 0.5 mg intravenous and a 2.5 mg oral dose are shown in Fig. 3.

The intravenous data were best described by a 2 compartment model and consequently the verapamil concentration-time profile was fit to the following bioexponential equation \(^{23}\) using a nonlinear least squares computer program (NONLIN); \(^{24}\)

\[
C = Ae^{-\alpha t} + Bo^{-\beta t}
\]

where C is the concentration of verapamil in blood; A and B are intercepts; and \(\alpha\) and \(\beta\) represent hybrid first order rate

TABLE II
Interday Assay Variability of Verapamil and Norverapamil in Blood
(Nine determinations for each concentration)

<table>
<thead>
<tr>
<th>Verapamil Conc.(ng/ml)</th>
<th>Coefficient of Variation (%)</th>
<th>Norverapamil Conc.(ng/ml)</th>
<th>Coefficient of Variation (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>23.6</td>
<td>6.18</td>
<td>23.8</td>
<td>4.70</td>
</tr>
<tr>
<td>157.5</td>
<td>7.16</td>
<td>159.0</td>
<td>9.19</td>
</tr>
<tr>
<td>787.5</td>
<td>3.18</td>
<td>795.0</td>
<td>4.28</td>
</tr>
</tbody>
</table>
Concentration-time profiles of verapamil following intravenous and oral administration. The circles depict the observed concentrations of verapamil and the solid lines denote the best fit curve to those points. The open triangles represent the norverapamil levels observed after oral dosing. Norverapamil could not be detected following intravenous verapamil administration.
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constants for distribution and elimination, respectively. The terminal half-life of verapamil in this rat was 1.42 hours.

Following oral administration, verapamil concentrations were characterized by a two compartment model with a first-order rate of absorption described by the following equation:

\[ C = A'e^{-ka \cdot t} + B'e^{-\alpha t} + C'e^{-\beta t} \]

where \( C \) is the concentration of verapamil in blood; \( A' \), \( B' \) and \( C' \) represent the intercepts obtained by log-peeling of the concentration time curve; and \( ka \), \( \alpha \) and \( \beta \) represent first order rate constants for absorption, distribution and elimination, respectively. The terminal half-life of verapamil after oral dosing was 3.14 hours. As in man, \(^{25-26}\) norverapamil blood levels were comparable to verapamil concentrations following oral administration, but could not be detected after the intravenous verapamil dose. Due to the limited blood sampling, no mathematical description of norverapamil blood levels was attempted.

The areas under the verapamil concentration-time curves (AUC) following intravenous and oral administration were determined by the trapezoidal method with extrapolation to infinite time from the last concentration time point.\(^{27}\) This information was then used to calculate the oral availability (\( F_o \)) of verapamil by the following equation:\(^{28}\)

\[ F_o = \frac{D_{iv} \cdot AUC_{o} \cdot t_{iv}}{D_{o} \cdot AUC_{iv} \cdot t_{o}} \]
where $AUC_0$ and $AUC_{iv}$ represent the areas under the concentration-time profiles of verapamil following oral and intravenous verapamil administration, respectively; $D_{iv}$ and $D_o$ denote the intravenous and oral dose administered; and $t_{iv}/t_o$ is the correction factor due to differences in verapamil half-life following intravenous and oral dosing. The estimated oral availability of verapamil was 3.2%.

This work demonstrates the importance of a sensitive and selective analytical assay for verapamil studies in rat where sampling volumes are limited. It also suggests the rat may be a useful animal model for future studies concerned with the route of administration on verapamil systemic availability.

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