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Report

High-Performance Liquid Chromatographic (HPLC) Assay Using Fluorescence Detection for the Simultaneous Determination of Gallopamil and Norgallopamil in Human Plasma

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Gallopamil is a calcium-channel antagonist with reported activity in experimental animals three to five times higher than that of verapamil. An automated high-performance liquid chromatographic (HPLC) method with fluorescence detection is described for the simultaneous determination of gallopamil and its metabolite norgallopamil in plasma. Gallopamil was well resolved from norgallopamil and other metabolites, allowing simultaneous quantitation of both drugs. The detection limit for both gallopamil and norgallopamil was 0.9 ng/ml. This method has been successfully used for the determination of gallopamil and norgallopamil following the administration of 25-, 37.5-, and 50-mg oral doses of drug.

KEY WORDS: gallopamil; D-600; norgallopamil; norverapamil; high-performance liquid chromatography (HPLC); fluorescence detection.

INTRODUCTION

Gallopamil {α-isopropyl-α-[(*N*-methylhomoveratryl)-γ-aminopropyl]-3,4,5-trimethoxyphenyl-acetonitrile} is a methoxy analogue of verapamil (Fig. 1). Both compounds are calcium antagonists; gallopamil is currently available in Europe and is undergoing clinical trials in the United States for the treatment of angina and hypertension. Studies carried out in West Germany have elucidated that, analogous to verapamil, gallopamil undergoes extensive first-pass metabolism, resulting in low plasma concentrations of drug and a large number of N-demethylated and O-demethylated metabolites (1).

The quantitation of verapamil in plasma has been investigated by gas chromatography with nitrogen phosphorous detection (2,3) and, more extensively, by high-performance liquid chromatography (HPLC) with fluorescence detection (4–8). No method has been published pertinent to the analysis of gallopamil or its metabolite, norgallopamil, in plasma. In order to study the pharmacokinetics of gallopamil and correlate the pharmacologic effect with plasma concentrations, it is necessary to develop a sensitive analytical method that is suitable for application to a large number of blood samples.

The automated HPLC procedure described in this report uses fluorescence detection and employs an expedient sample preparation method. It is suitable for pharmacokinetic studies after single or multiple dosing. This procedure allows the simultaneous quantitation of gallopamil and the N-desmethyl metabolite norgallopamil; other known metabolites of gallopamil do not interfere. The limit of detection of this HPLC fluorescence detection procedure is <1 ng/ml for both drug and metabolite. The utility of the method for studying the pharmacokinetics was demonstrated by the determination of gallopamil and norgallopamil levels after a single oral administration of 50 mg of gallopamil to healthy subjects.

MATERIALS AND METHODS

Solvents, Reagents, and Glassware

Unless otherwise stated, all solvents and reagents used were HPLC grade. Acetonitrile, hexane, methyl-t-butyl ether, methanol, and toluene were from Burdick & Jackson (Muskegon, Mich.); triethylamine, potassium dihydrogen phosphate (ACS grade), and potassium hydroxide (ACS grade) were supplied by Fisher (Fair Lawn, N.J.); and 1-octanesulfonic acid was obtained from Kodak (Rochester, N.Y.). Gallopamil and norgallopamil were supplied as hydrochloride salts by Knoll A.G. (Ludwigshafen, West Germany); verapamil and norverapamil were provided as hydrochloride salts by Knoll Pharmaceutical (Whippany, N.J.). Disposable glass culture tubes (16 × 125 mm) and disposable conical centrifuge tubes (16 × 125 mm) were obtained from American Scientific Products (McGaw Park, Ill.). The

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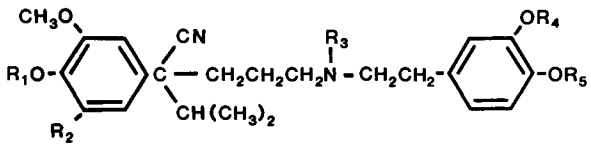

SUBSTANCE	R_1	R_2	R_3	R_4	R_5
Gallopamil	CH_3	OCH_3	CH_3	CH_3	CH_3
Norgallopamil	CH_3	OCH_3	H	CH_3	CH_3
Verapamil	CH_3	H	CH_3	CH_3	CH_3
Norverapamil	CH_3	H	H	CH_3	CH_3
PR-53	CH_3	OCH_3	CH_3	CH_3	H
PR-54	CH_3	OH	CH_3	CH_3	CH_3
SZ-488	CH_3	OCH_3	CH_3	H	CH_3
SZ-524	CH_3	OCH_3	H	CH_3	H

Fig. 1. Chemical structures of gallopamil, norgallopamil, verapamil, norverapamil, and several gallopamil metabolites.

conical centrifuge tubes were silanized with 10% dichlorodimethylsilane (Kodak) in toluene.

Standard and Internal Standard Preparation

Stock standard solutions of gallopamil, norgallopamil, and norverapamil were prepared at the respective concentrations of 100, 50, and 60 μg/ml expressed as the free bases. These solutions were stored in amber, silanized volumetric flasks at 5°C and showed no deterioration after 3 months. Solutions used for the preparation of plasma standards were prepared from the stock solutions and used fresh. The internal standard, norverapamil, was diluted to 60 ng/ml with distilled water.

Instrumentation and Chromatographic Conditions

The chromatography system was comprised of a Waters 840 system (Millipore, Cary, N.C.) with 510 pumps, WISP, and a McPherson FL-749 fluorometer (S.I. McPherson, Acton, Mass.) fitted with a 24-μl flow cell. Chromatography was carried out at 40°C using a water-jacketed 4.6 mm × 25-cm, 5-μm Supelcosil LC-18-DB analytical column and a 2-cm precolumn containing the same packing material. The mobile phase was 38% acetonitrile:62% aqueous phase (v:v). The aqueous phase consisted of 50 m*M* KH_2PO_4, 5 m*M* 1-octanesulfonic acid, 1 m*M* triethylamine and was adjusted to pH 3.0 with phosphoric acid. The flow rate of the mobile phase was 2 ml/min. Column efficiency was routinely monitored utilizing the peak width at half-height method (9).

The excitation and emission spectra of gallopamil (8.33 μ*M*), norgallopamil (4.24 μ*M*), and verapamil (8.35 μ*M*) were obtained using the McPherson fluorometer, which was fitted with a cuvette assembly. The detector excitation wavelength was 205 nm and the emitted fluorescence was monitored through a 0-56 filter (Kopp, Pittsburgh, Pa.) fitted into the high-sensitivity accessory (McPherson, Acton, Mass.). The gain, time constant, and range settings on the fluorometer were 930 V, 5, and 0.1, respectively. Several other glass filters were evaluated. These filters were 9-54 (Kopp) and WG305, WG295, and WG280 (Schott, Duryea, Pa.).

Extraction Procedure

The internal standard, norverapamil, was added as 100 μl of a 60 ng/ml solution to each 1-ml aliquot of plasma in a glass culture tube (with a Teflon-lined screw cap). To adjust the plasma pH to 13, 0.1 ml of 2 *M* KOH was added. The plasma was vortexed gently for 5 sec to mix the components. The samples were extracted with 5 ml of a solvent mixture consisting of hexane:methyl-t-butyl ether (4:1). Mixing was accomplished by rotating the tubes on a shaker (Glas-Col Apparatus Co., Terre Haute, Ind.) at 45 rpm for 30 min. After centrifugation, at 3000 rpm (1150*g*) for 5 min at room temperature, the samples were frozen for 20 min at −80°C in a Revco freezer. Immediately upon removing the tubes from the freezer, the organic layer was decanted into a silanized 15-ml disposable conical tube. The organic solvent was back extracted at ambient temperature with 0.1 ml of 50 m*M* KH_2PO_4 (pH 3) by mechanical shaking (Eberbach shaker, Eberbach Corp., Ann Arbor, Mich.) at high speed for 5 min. The tubes were centrifuged for 5 min and frozen at −80°C for 20 min; the organic layer was then discarded. The aqueous layer was transferred to glass WISP vials by inserting a 100-μl Gilson pipette into the aqueous extract and withdrawing 95 μl. Careful attention was taken to avoid contamination with any residual organic solvent.

Validation Procedures

The absolute recoveries of gallopamil and norgallopamil were measured for six replicates at each of three concentrations (5, 25, and 50 ng/ml). The recoveries were determined by comparing the peak height values of extracted plasma samples with the peak height values for a standard solution.

The intra- and interday accuracy and precision of the method were assessed over the range 0.9–94.4 and 0.9–46.4 ng/ml for gallopamil and norgallopamil, respectively. The intraday data were obtained from the analysis of replicate samples (N = 6) fortified with 0.91, 1.82, 45.7, and 91.4 ng/ml gallopamil and 0.93, 23.2, and 46.4 ng/ml norgallopamil. Interday assay precision and accuracy were determined at these same concentrations and also at 4.57 ng/ml gallopamil and 2.32 ng/ml norgallopamil. The best-fit straight line for the calibration standards was routinely determined using weighted least-squares regression analysis (10).

RESULTS

Detector and Chromatography Optimization

Prior to HPLC analysis, the fluorescence excitation spectra (Fig. 2A) of gallopamil and norgallopamil were determined in the HPLC mobile phase using the cuvette assembly. Two well-resolved maxima at 200 and 228 nm were observed for both drugs. The excitation wavelength used for HPLC analysis was further optimized by making replicate injections of a standard gallopamil solution on the column while varying the excitation wavelength within the range of 200–207 nm. A comparison of the peak heights indicated that the maximum sensitivity was obtained at 205 nm.

The emission spectra of the drug and metabolite are

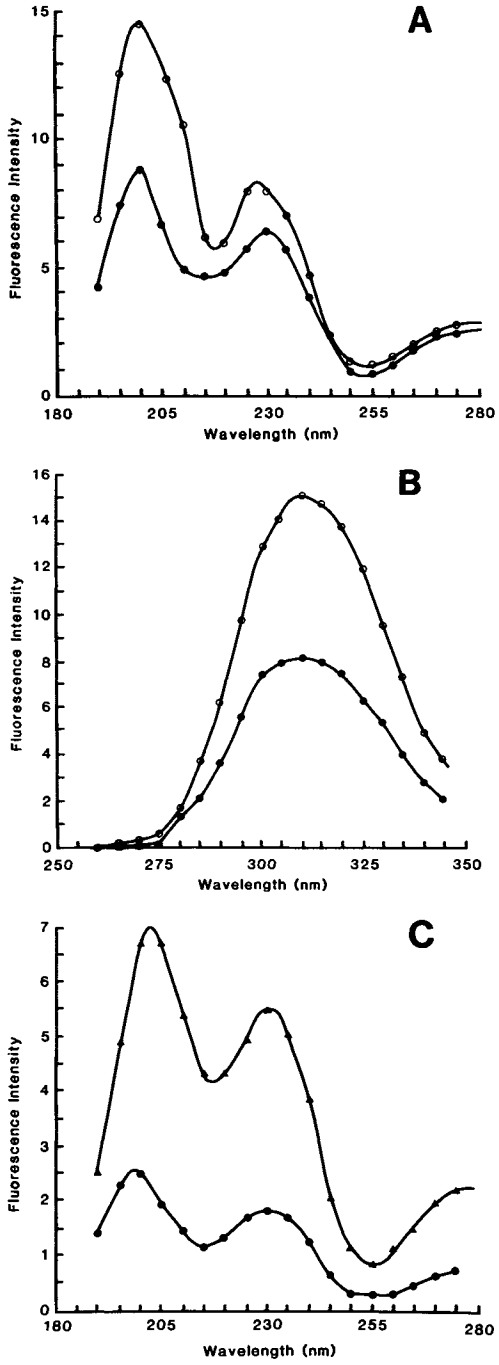

Fig. 2. Fluorescence spectra of gallopamil (filled circles), norgallopamil (open circles), and verapamil (filled triangles). The units of the fluorescence intensity are normalized for concentration. (A) Excitation spectra of gallopamil and norgallopamil as determined in the mobile phase. (B) Emission spectra of gallopamil and norgallopamil. (C) Excitation spectra of gallopamil relative to verapamil.

shown in Fig. 2B. The maximum emission intensity observed for gallopamil and norgallopamil occurred at 310 and 313 nm, respectively. Several glass filters (WG280, WG295, WG305, 0-56, and 9-54) were identified as potential emission filters. The 0-56 and WG280 filters gave the best sensitivity and were equivalent. The 0-56 filter was used for all analytical work described.

Gallopamil Chromatography and Assay Validation

Ion-pair chromatography allowed the resolution of gallopamil, norgallopamil, and endogenous components and provided 25,000–30,000 plates/meter (for gallopamil). Representative chromatograms are shown in Fig. 3. Figure 3A illustrates a chromatogram of blank plasma; Fig. 3B shows plasma spiked with 0.9 ng/ml norgallopamil (D845), 1.8 ng/ml gallopamil (D600), and internal standard (NORV). Retention times for drug, metabolites, and several other drugs which could be coadministered with calcium antagonists are summarized in Table I.

The intraday accuracy and precision of the assay were determined by the analysis of replicate plasma spikes at four different plasma concentration levels for gallopamil and three levels for norgallopamil (Table II). The precision showed a maximum CV of 18% for gallopamil and 15% for norgallopamil at the detection limit of 0.9 ng/ml.

The interday accuracy and precision data of the analytical method are shown in Table III. The values for accuracy

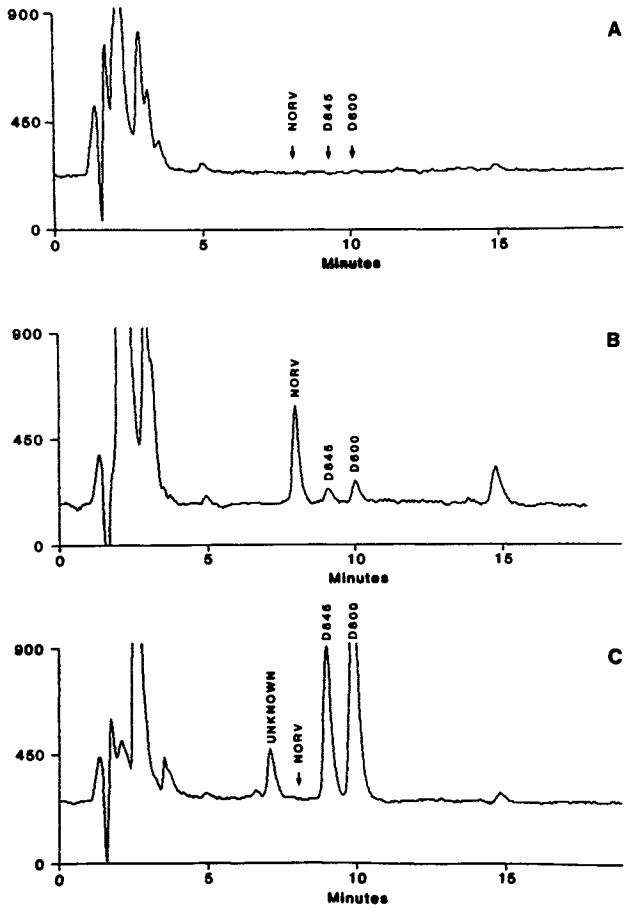

Fig. 3. Representative chromatograms of plasma extracts. (A) Blank plasma. (B) Blank plasma containing 1.8 ng/ml gallopamil (D600), 0.9 ng/ml norgallopamil (D845), and the internal standard (NORV). (C) Plasma extract from an angina patient who was administered gallopamil (37.5 mg tid) to steady state; plasma contained 23.1 ng/ml gallopamil and 13.6 ng/ml norgallopamil.

Table I. Retention Times of Gallopamil, Gallopamil Metabolites, and Several Other Drugs Which May Be Coadministered to Patients Receiving Gallopamil

Drug	Retention time (min)
Gallopamil (D-600)	10.1
Norgallopamil (D-845)	9.2
Norverapamil	8.0
D-517	7.5
PR-53	6.8
PR-54	6.0
SZ-488	7.5
SZ-524	6.3
Hydrochlorothiazide	1.8
Propranolol	3.8
Triamterene	1.9
Quinidine	2.3
Procainamide	1.6

or analytical bias are all within 100 ± 10%. As expected, the coefficients of variation are similar or slightly higher than those reported for the interday data. The absolute recoveries of gallopamil and norgallopamil were determined over a range of 5 to 50 ng/ml. The recovery was 81.1 ± 9.0% for gallopamil and 63.1 ± 9.1% for norgallopamil (N = 18).

DISCUSSION

Although verapamil has been extensively investigated by HPLC utilizing fluorescence detection (4–8), very little information regarding the actual fluorescence spectra is available in published assays. These investigators utilize different excitation wavelengths without justifying why each was chosen. Kuwada *et al.* (5) and Kapur *et al.* (7) used an excitation wavelength of 203 nm; Lim *et al.* (6) reported an excitation wavelength of 278 nm, while Kacprowicz *et al.* (8) reported used 280 nm. Although all of these investigators recorded using a similar emission wavelength of about 320 nm, the excitation wavelengths were either 203 or 280 nm. Inspection of Fig. 2C shows that verapamil has three excitation maxima (205, 228, and 280 nm); each of these has been separately reported and used in the literature. The excitation spectra for gallopamil and norgallopamil are qualitatively similar to those for verapamil (Figs. 2A and C); however, at the excitation wavelength of 205 nm, the intensity of emitted fluorescence for verapamil is three times greater than that observed for gallopamil (Fig. 2C). Interestingly, the fluorescence intensity of gallopamil is approximately one-half that of its metabolite, norgallopamil, when measured by either the excitation (Fig. 2A) or the emission (Fig. 2B) spectra.

Table II. Intraday Assay Precision and Accuracy Data Obtained for Gallopamil and Norgallopamil in Plasma

Drug	Concentration (ng/ml)		Accuracy (%)	CV (%)
	Actual	Measured[a]		
Gallopamil	0.91	1.03	113	18
Gallopamil	1.82	1.81	99	8
Gallopamil	45.7	43.3	95	2
Gallopamil	91.4	86.1	94	4
Norgallopamil	0.93	0.88	95	15
Norgallopamil	23.2	22.7	98	3
Norgallopamil	46.4	44.3	96	4

[a] Mean concentration of six replicate samples.

Table III. Interday Assay Precision and Accuracy Data Obtained for Gallopamil and Norgallopamil in Plasma

Drug	Concentration (ng/ml)		Accuracy (%)	CV (%)	N
	Actual	Measured[a]			
Gallopamil	0.91	0.99	109	17	17
Gallopamil	1.82	1.93	106	11	18
Gallopamil	4.57	4.56	100	9	23
Gallopamil	45.7	45.9	100	8	41
Gallopamil	91.4	87.6	96	7	18
Norgallopamil	0.93	0.97	102	19	18
Norgallopamil	2.32	2.34	101	11	23
Norgallopamil	23.2	23.5	101	5	41
Norgallopamil	46.4	46.0	97	8	18

[a] Mean concentration of N replicates.

The dose of gallopamil (25–50 mg) under investigation is lower than that generally used for verapamil (80–120 mg). The combination of a reduced dosage regimen (approximately two- to threefold) resulting in lower plasma concentrations and diminished fluorescence (threefold) of gallopamil relative to verapamil presented a challenge to the sensitivity of instrumentation currently available. The required assay sensitivity was achieved by optimization of sample quality, detector optics, and chromatography mobile-phase conditions.

The sample preparation procedure is somewhat analogous to that reported for meperidine (11), urapidil (12), and verapamil (3). The back extraction of the initial hexane–ether extract using the aqueous component (pH 3, 50 mM KH_2PO_4) of the mobile phase concentrated the analytes and at the same time provided a highly purified sample, allowing automated analysis of a large number of samples per day.

An analogue of verapamil (D-517) was initially used as the internal standard for this assay. This candidate was chosen because of structural similarities to gallopamil; also, it has been used successfully as an internal standard for verapamil/norverapamil assays. Although the use of D-517 showed excellent accuracy and reproducibility, it was later discovered to co-chromatograph (Fig. 3C) with an unknown gallopamil metabolite which was present in subjects dosed with gallopamil. It should be noted, however, that the area around norverapamil did not contain any other peaks. Therefore, norverapamil was used as an internal standard. One limitation of using norverapamil is that the gallopamil assay cannot be used in patients being treated concurrently with verapamil. The concomitant use of both agents, however, is highly unlikely.

This method has been successfully used for the determination of gallopamil and norgallopamil in human plasma following the administration of single and multiple doses of 25, 37.5, and 50 mg. A representative plasma concentration vs time curve of one subject, who was administered a 50-mg tablet, is shown in Fig. 4 as an illustration of the applicability

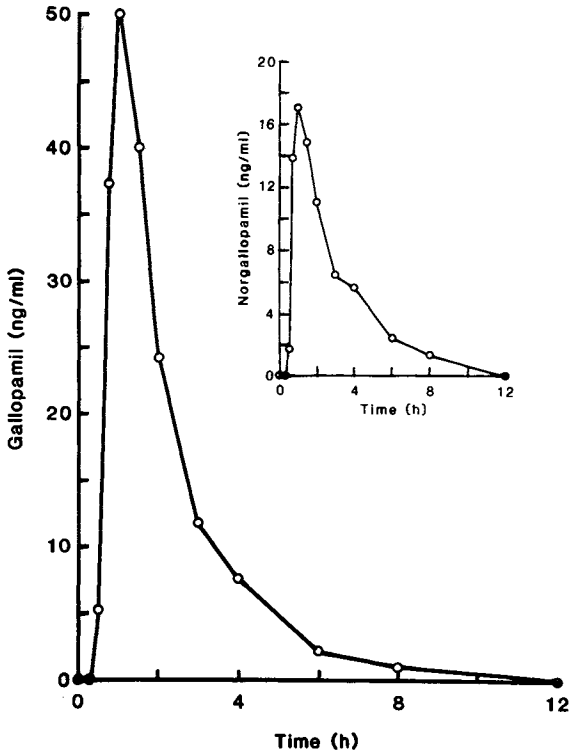

Fig. 4. Representative plasma gallopamil concentration versus time curve for one subject who was administrated a single 50-mg tablet of gallopamil. Inset shows the concentration of norgallopamil as a function of time.

of this assay. The peak time for both gallopamil and norgallopamil was 0.75 hr. The half-lives of gallopamil and norverapamil were 2.5 and 2.7 hr, respectively.

In summary, a specific, sensitive, and precise HPLC assay for both gallopamil and norgallopamil is described. The assay described is capable of detecting <1 ng/ml and there are no interferences from other gallopamil metabolites.

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