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Note

Analysis of verapamil in plasma by liquid chromatography

T.M. JAOUNI, M.B. LEON, D.R. ROSING and H.M. FALES*

Laboratory of Chemistry and Cardiology Branch, NHLBI, Bethesda, MD 20205 (U.S.A.)

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Verapamil hydrochloride [DL-2,8-bis(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile hydrochloride, Isoptin®], a drug which inhibits calcium flux across cell membranes, has been shown to have anti-anginal, anti-hypertensive, and anti-arrhythmic properties [1]. More recently, verapamil has been used as an alternative to propranolol in treating patients with hypertrophic cardiomyopathy [2—4]. Effective therapy would be greatly assisted by analysis of verapamil in plasma or whole blood. Known metabolites of verapamil, except for its N-demethyl derivative (D-591), are of much lower potency and of no clinical importance [5]. Verapamil has been analyzed previously by fluorometry [6], gas chromatography [7—10] and selected ion monitoring (mass fragmentography) [11]. Direct fluorometry may not be specific since some of the known metabolites (norverapamil, etc.) have similar spectra. Gas chromatography and mass spectral selected ion monitoring require internal standards, special detectors or both. In the case of the latter, the apparatus is complex and costly.

Recently, Harapat and Kates [12] reported a simple and sensitive liquid chromatographic procedure using a commercially available column and fluorometric detection for the analysis of verapamil in plasma and blood. Unfortunately, our attempt to reproduce the procedure using the recommended column and solvent system was unsuccessful; neither verapamil nor the internal standard (D-517) eluted within 3 h. Adjustment of the solvent system to 70% methanol—0.01 *M* 2-propanesulfonic acid sodium salt—0.15% glacial acetic acid did cause the compounds to elute in 16 min but resolution and peak shape were poor. A second column, generously provided by the same manufacturer, was no better in this regard, although excellent peak shapes were observed with the recommended non-basic test compounds, naphthalene and biphenyl. Accordingly, we sought alternative columns and conditions.

*To whom correspondence should be addressed.

MATERIALS AND METHODS

Materials

DL-Verapamil hydrochloride, its N-demethyl derivative (D-591), a methoxy analogue [2-(3,4,5-trimethoxyphenyl)-8-(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile hydrochloride, D-600], and a lower homologue [2,7-bis(3,4-dimethoxyphenyl)-5-methyl-2-isopropyl-5-azaheptanitrile hydrochloride, D-517] were all gifts from Knoll Pharmaceutical (Darmstadt, G.F.R.). The internal standards were stored in the mobile phase at 0°C. Acetic anhydride was purchased from Allied Chemical (Morristown, NJ, U.S.A.), solvents from Burdick and Jackson Labs. (Muskegon, MI, U.S.A.), tetramethylammonium chloride from Eastman Kodak (Rochester, NY, U.S.A.) and monobasic potassium phosphate from Mallinckrodt (St. Louis, MO, U.S.A.). Water was distilled and deionized by the Milli-Q System (18 megohm) (Millipore, Bedford, MA, U.S.A.).

Liquid chromatography

A Spectra-Physics Model 3500 liquid chromatograph and a Schoeffel Model FS 970 fluorometer were used for all analyses. The fluorometer was set at an excitation wavelength of 200 nm. The glass envelope of the phototube and a Corning 5970 filter (5.06 mm thick) allowed fluorescence (67% transmission) between 310 and 390 nm to be detected by the phototube. The detector range was varied between 0.2 and 0.5 μA full scale depending on concentration, and the time constant set at 6 sec. The fluorometer electronics were allowed to remain on constantly; only the ultraviolet source was turned off.

The chromatographic system finally found most useful was a LiChrosorb RP-18 (10 μm) Hibar II (EM Labs., Cincinnati, OH, U.S.A.) run at a flow-rate of 1.8 ml/min at 1210 p.s.i. inlet pressure. The isocratic mobile phase was 40% (v/v) acetonitrile—water, 0.01 *M* in tetramethylammonium chloride, 0.005 *M* in monobasic potassium phosphate and 0.074 *M* in phosphoric acid. The solution was degassed for at least 5 min and maintained under bubbling helium. The column was washed thoroughly with water and then with methanol at the end of each run. At the start of each run the column was washed with the mobile phase while the ultraviolet lamp of the fluorometer was allowed to stabilize.

All glassware was carefully cleaned with sulfuric—dichromate solution and washed extensively with distilled water, acetone and diethyl ether.

Hamilton microliter syringes were used for all quantitation.

Extraction procedure

The extraction procedure was essentially the same as that used by Harapat and Kates [12]: To 0.5 ml of plasma in a 15 × 125 mm glass test tube were added either 100 or 400 ng of D-600 internal standard depending on whether the verapamil was expected to be in the range of 10—100 or 100—500 ng/ml plasma. When the sample was found to be outside of the appropriate range the analysis was repeated, adjusting the quantity of internal standard. Twenty-five μl of 5 *N* sodium hydroxide were added, the tube agitated gently, 5 ml of diethyl ether added and the sample was vortex-mixed for 1 min. After a further minute of centrifuging, most of the top layer was carefully pipetted into a 120-

mm tube whose base had been elongated to accommodate ca. 50 μl. Acetic anhydride (25 μl) was then added to the diethyl ether to react with the dealkylated metabolites, the tube vortex-mixed for 15 sec and allowed to stand at room temperature for 5 min.

Fifty μl of 0.12 *M* sulfuric acid was then added and the tube vortex-mixed for 1 min and then centrifuged for 1 min in a receiver designed to protect the tube's coned tip. Depending upon the amount of verapamil expected, 6—25 μl of the aqueous layer were withdrawn with a syringe and injected into the chromatograph.

RESULTS AND DISCUSSION

An RP-18 (10 μm) Hibar II column (250 mm × 4.6 mm I.D.) (EM Labs.) with tetramethylammonium chloride—phosphate buffer—acetonitrile mobile phase [9, 13, 14], as suggested by Dr. G. Burce, finally provided excellent results with respect to separation of verapamil and either of two available internal standards. However, the separation between verapamil and its known major metabolite, N-demethylverapamil (D-591), while sufficient for qualitative identification (Fig. 1, before acetylation) interferes with quantitation of the parent compound. Treating the ethereal extract from plasma with acetic anhydride converts the metabolite to its N-acetyl derivative, ensuring that it will not be extracted with verapamil in the subsequent acid treatment (Fig. 1, after acetylation). The N-acetyl derivative elutes considerably later (18 min) with the same mobile phase and could be determined separately if desired. The detector

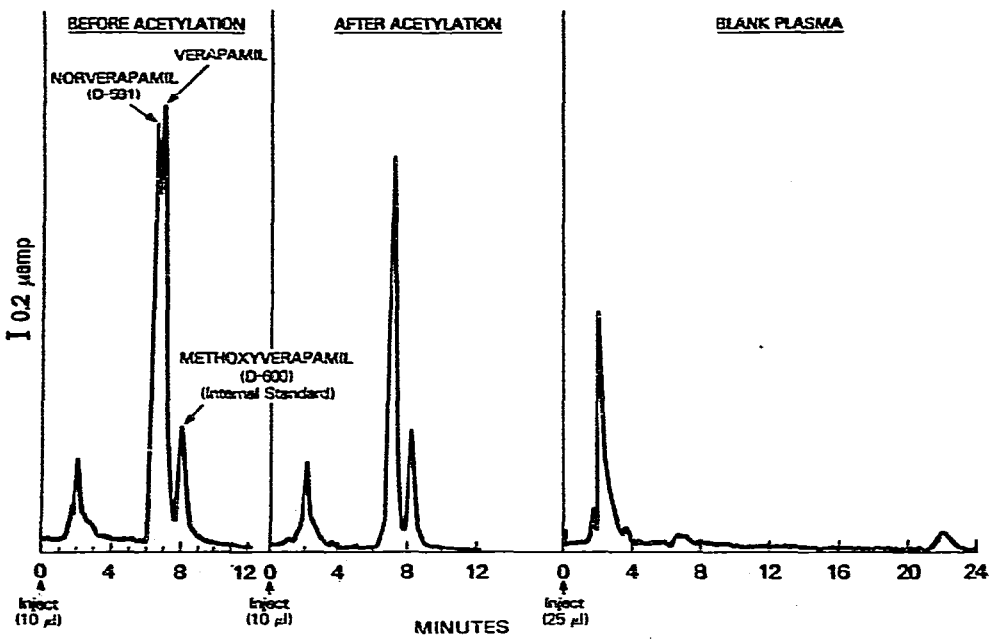

Fig. 1. Liquid chromatogram of 10-μl aliquots taken from 50 μl total extract of 500 μl of plasma containing 200 ng/ml of each component, showing effect of addition of acetic anhydride. Blank plasma, 25 μl of a similar extraction, is shown for reference.

response (peak area per mole) of N-acetyldemethylverapamil is approximately the same as demethylverapamil and verapamil under these conditions. Collection of the peak provided a sample for mass spectrometry by direct insertion probe. Important ions are observed at *m/z* 482 ($M^{+\cdot}$, 5), 289 [$M^{+\cdot}$–ketene–$(H_3CO)_2C_6H_4CH_2$, 52] and 164 [$(H_3CO)_2C_6H_4CH{=}CH_2$, 100].

Other primary and secondary amine metabolites of verapamil have been noted by McIlhenny [7] and Neugebauer [5]; these would likewise be removed by acetic anhydride. Tertiary amine metabolites [15] include 2-(3,4-dimethoxyphenyl)-5-dimethylamino-2-isopropylpentanitrile (metabolite XI), conjugates of a phenolic analogue of metabolite XI (metabolite XII) and an O-demethylverapamil [2-(4-hydroxyphenyl)-8-(3,4-dimethoxyphenyl)-6-methyl-2-isopropyl-6-azaoctanitrile (metabolite III)]. The first two are present in urine in trace quantities and because of their grossly different structures might be expected to separate easily from verapamil on chromatography. The latter accounts for 7% of the dose excreted in urine but since it is a conjugate it would not be extracted under the above conditions.

With most plasma samples we have encountered a very small conflicting peak eluting approximately at the same retention volume as verapamil (Fig. 1, blank plasma), but it has not yet exceeded a quantity equivalent to ca. 0.1 ng verapamil per ml plasma.

Standard curves were prepared using spiked plasma in two ranges, 0—100 ng/0.5 ml and 0—500 ng/0.5 ml. Excellent linearity was achieved in both cases (correlation coefficient=0.999) with the plots extrapolating through the origin.

Table I shows the coefficient of variation in replicate plasma analyses (n=6). Best results are obtained with peak height ratios of verapamil to D-600 between 0.8 and 3.2.

TABLE I

REPRODUCIBILITY AT A GIVEN PLASMA CONCENTRATION

n = 6.

Peak height ratio of verapamil to D-600 (mean)	C.V. (%)	Verapamil (ng/0.5 ml plasma)	Internal standard added (ng D-600)
0.37	3.1	10	100
0.86	1.7	100	400
1.59	1.6	50	100
2.14	1.2	250	400
3.16	1.4	500	400
3.66	3.6	100	100

The technique is currently being applied in these laboratories for the clinical analysis of verapamil in man at levels of 5—600 ng/ml, although the method has been found to be satisfactory at levels as low as 1 ng/ml. Preliminary clinical studies were performed in the cardiac catheterization laboratory to assess electrophysiologic responses to intravenous verapamil in eight patients with hypertrophic cardiomyopathy. Plasma verapamil levels for the group (mean ± S.E.M.) after 0.007 mg/kg/min, 0.014 mg/kg/min, and 0.021 mg/kg/min constant infusions, each preceded by 0.1 mg/kg intravenous bolus injections, were 144 ± 18

ng/ml (range 74—183 ng/ml), 319 ± 45 ng/ml (range 154—540 ng/ml), and 415 ± 13 ng/ml (range 164—560 ng/ml), respectively. Further studies, analyzing verapamil levels in patients on chronic oral therapy are currently in progress.

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