ELSEVIER

Journal of Chromatography B, 667 (1995) 349–354

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Short communication

Simultaneous analysis of verapamil and norverapamil enantiomers in human plasma by high-performance liquid chromatography☆

Grazia Stagni*, William R. Gillespie[1]

Pharmaceutics Division, College of Pharmacy, The University of Texas at Austin, Austin, TX 78712, USA

First received 12 October 1994; revised manuscript received 2 January 1995; accepted 5 January 1995

Abstract

An improved HPLC method for the simultaneous determination of the enantiomers of verapamil (V) and its major metabolite norverapamil (NV) in human plasma samples is presented. NV is acetylated immediately to N-acetylnorverapamil (ANV) in the extraction solvent (2% butanol in hexane). Acetylation is so rapid that it does not delay sample processing. ANV and V enantiomers are then separated on an α_1-acid glycoprotein chiral column with a mobile phase of phosphate buffer (0.01 *M*, pH 6.65) and acetonitrile. The fluorescence detector wavelengths are set at 227 nm for excitation and 308 nm for emission. Introduction of the internal standard (I.S.) (+)-glaucine improves accuracy, precision and robustness of the method. The assay is sensitive and specific. Baseline separation is achieved for both V and ANV. Limits of quantitation are 3 ng/ml for V and 2 ng/ml for NV (single enantiomer) with precision and accuracy better than 15% at those levels. Detector response is linear in the range tested (3–200 ng/ml for V and 2–100 ng/ml for NV, single enantiomer). This assay has been applied to a clinical study of the pharmacodynamics of V involving six healthy volunteers.

1. Introduction

Verapamil (V) is a calcium channel blocking agent approved for treating hypertension, angina pectoris, and supraventricular tachyarrhythmias. V is clinically administered as a racemate, but the enantiomers differ substantially with respect to potency and pharmacokinetics [1–3].

This paper presents an improved assay for the direct determination of V and NV enantiomers in human plasma using a single α_1-acid glycoprotein chiral column. Following Hedman et al. [4] NV is acetylated to ANV. However, the derivatizing agent (acetic anhydride) is added directly to the extraction solvent (2% butanol in hexane) used to extract V and NV from plasma. Thus, unlike the previous method [4], acetylation is so rapid that the time required is almost negligible relative to the overall sample processing time. Also, an internal standard has been introduced to provide remarkable improvement in the preci-

☆ This work was presented in part at the American Association of Pharmaceutical Scientists 8th Annual Meeting, Orlando, FL, November 1993.

* Corresponding author. Current address: Division of Clinical Pharmacology, The University of Texas Health Science Center at San Antonio, 7703 Floyd Curl Drive, San Antonio, TX 78284, USA.

[1] Current address: Division of Biopharmaceutics, Food and Drug Administration, Rockville, MD 20857, USA.

0378-4347/95/$09.50
SSDI 0378-4347(95)00019-4

sion of the assay over that reported by Hedman et al. [4] for both V and NV. The assay also provides a lower limit of quantitation (LOQ) for NV than was reported by Shibukawa and Wainer [5] although the LOQ for V is about the same. On the other hand, the reported method has the advantages of using just a single column, an aqueous mobile phase, and complete processing at room temperature, but the disadvantage of a longer retention time. The method is simple and convenient for pharmacokinetic studies. Indeed, it has been applied to a three-way crossover single-dose study involving six health volunteers [6].

2. Experimental

2.1. Chemicals

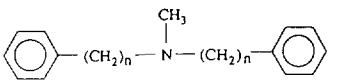
Racemic V hydrochloride and NV hydrochloride were a gift from Knoll Pharmaceutical Company (Whippany, NJ, USA). Single enantiomers of V and NV were obtained as a gift from Searle (Skokie, IL, USA). Internal standard (+)-glaucine was purchased from Aldrich (Milwaukee, WI, USA). Glass-distilled acetonitrile was purchased from Burdik and Jackson Labs. (Muskegon, MI, USA). The phosphate buffer (pH 6.65, 0.01 *M*) was prepared using potassium phosphate monobasic (A.C.S. Fisher Scientific Company, Fair Lawn, NJ, USA) and NaOH (J.T. Baker, Phillipsburg, NJ, USA). Hexane A.C.S., *sec.*-butanol certified and acetic anhydride A.C.S. were purchased from Fisher Scientific Company. Blank plasma was donated by the Austin Blood Bank.

2.2. Apparatus

The modular liquid chromatograph consisted of an LKB-Bromma 2150 HPLC pump (LKB Produkter AB, Bromma, Sweden), a Bio-Rad Model AS-100 HPLC automatic sampling system (Bio-Rad Laboratories, Richmond, CA, USA), a Hewlett-Packard HP 1046A programmable fluorescence detector and a Hewlett-Packard HP 3396A integrator (Hewlett-Packard, Avondale, PA, USA). The chiral column was a commercially available Chiral-AGP column (150 × 4.0 mm I.D.) and the guard column was a Chiral-AGP column (10 × 3.0 mm I.D.) from ASTEC (Whippany, NJ, USA). The internal standard was added using a Syva diluter dispenser (Palo Alto, CA, USA).

2.3. Internal standard selection

A suitable I.S. for this study was found by searching the Fine Chemical Directory (FCD) database using the MACCS-II software provided by Molecular Design Limited [7]. The topological query specified for the two-dimensional search was:



n = 1, 2, 3 or 4

Twenty-eight candidates were found. Six candidates were selected for testing based upon ease of commercial availability. (+)-Glaucine was selected because it had the most appropriate retention time. It interesting to note that this methodology leads to the same I.S. as chosen by Shibukawa and Wainer [5].

2.4. Chromatographic conditions

Chromatography was performed at room temperature. The flow-rate was 0.9 ml/min. The mobile phase was phosphate buffer (pH 6.65, 0.01 *M*)–acetonitrile (91:9, v/v). The excitation and the emission wavelengths of the fluorescence detector were set at 227 and 308 nm, respectively.

2.5. Sample preparation

To 1.0 ml of plasma in a screw-capped glass tube were added 50.0 μl of 2.5 μg/ml (+)-glaucine spiking solution, 100 μl of distilled water, and 200 μl of 2 *M* sodium hydroxide solution. Plasma samples were mixed and then added to 6 ml of hexane containing 2% *sec.*-

butanol. V and NV were extracted into the organic layer by vortex-mixing for 10 min. After centrifugation for 10 min at 1500 *g*, the aqueous layer was frozen in a dry ice–acetone bath and the hexane layer decanted into a clean glass tube. NV was acetylated to ANV by adding 50 μl of acetic anhydride directly to the decanted hexane layer. This organic phase was mixed and immediately evaporated to dryness under a dry nitrogen stream at room temperature. The resulting residue was reconstituted in 200 μl of a mixed solvent [potassium phosphate (0.01 *M*, pH 4.8)–acetonitrile (9:1, v/v)]. A 100-μl aliquot was injected onto the HPLC system.

2.6. Enantiomeric elution order

The elution order of the enantiomers of V and NV was determined by applying the method to unequal mixtures of the enantiomers.

2.7. Standards

Stock solutions of V (533.5 μg/ml), NV (317.5 μg/ml), and (+)-glaucine (482.3 μg/ml) were prepared in distilled water and stored in aliquots of 200 μl at −20°C. On each day of validation or clinical sample analysis, spiked solutions were prepared by diluting aliquots of the stock solutions to the desired concentrations. A single spiked solution was prepared containing both V and NV.

2.8. Extraction efficiency and recovery

Absolute recovery was determined by comparing the peak height for extracted samples with that of unextracted samples of identical concentration. Two concentration levels, a low (10.53 ng/ml V racemate, 10.05 ng/ml NV racemate) and a high (105.3 ng/ml V racemate and 100.5 ng/ml NV racemate) in the expected range of V and NV plasma levels were investigated. The extraction efficiency of the I.S. was determined at the working concentration (120.6 ng/ml).

2.9. Derivatization efficiency

A 1-ml volume of plasma was spiked with 0, 10.05, 20.10, 40.20, 100.5, 201.0, 301.5, 445.0 ng of NV and processed by the proposed procedure. Two replicates for each level of drug were analyzed for NV and ANV. After derivatization all chromatograms showed a completely flat baseline at the retention times of NV enantiomers.

2.10. Sample stability in the autosampler

Sample stability in the autosampler was determined by injecting six samples of different concentrations and then injecting the same samples again after 12 h, keeping them at 5°C between injections. No differences in the peak-height ratio or retention times were detected between the two series of injections, proving that there was no significant degradation of V during residence in the autosampler.

3. Results and discussion

3.1. Chromatographic separation

Fig. 1B shows a chromatogram of a plasma sample spiked with V and NV enantiomers. All the peaks have a baseline separation with a relative retention $\alpha \geq 1.2$. Resolution was $R_{(R\text{-ANV},S\text{-ANV})} = 2.3$, $R_{(R\text{-V},S\text{-V})} = 3.3$, $R_{(S\text{-ANV},\text{I.S.})} = 2.6$, $R_{(\text{I.S.},R\text{-V})} = 3.9$.

The mobile-phase composition was slightly modified from the one proposed by Hedman et al. [4] in order to accommodate the I.S. Small changes in the pH of the buffer can dramatically compromise the separation. With a protein-based stationary phase, pH affects the ionization and, possibly, the conformation of the protein, not only the ionization state of the analyte. Decreasing the pH decreases the resolution of the ANV enantiomers, increasing the pH decreases the resolution between *S*-ANV and the I.S.

Another factor affecting separation is the solvent mixture used to reconstitute the sample to inject it onto the column. We observed that

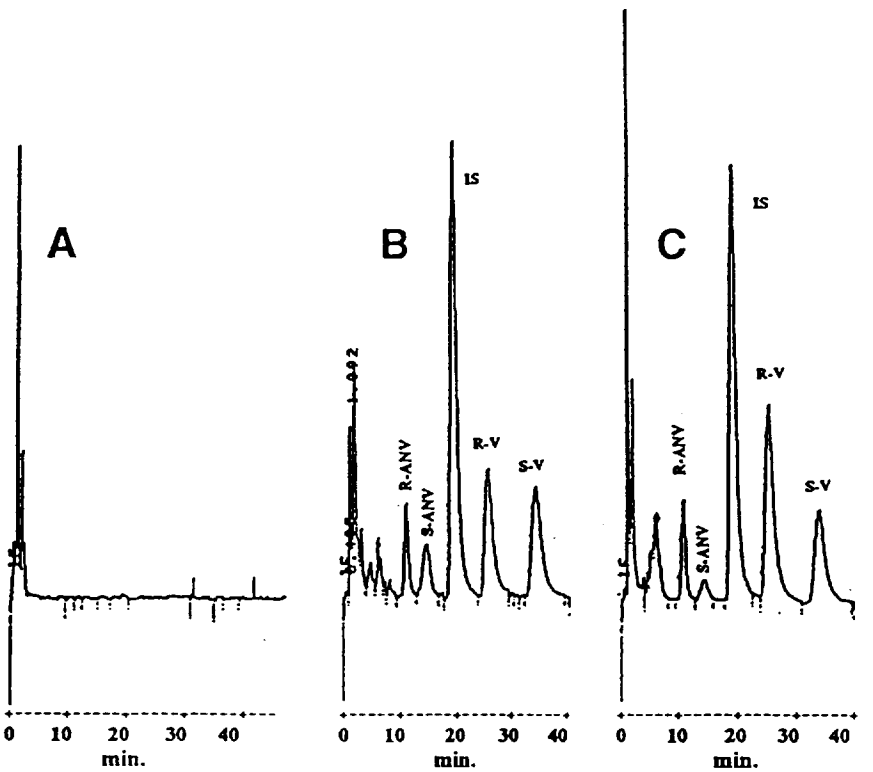


Fig. 1. HPLC separation of plasma extracts derivatized with acetic anhydride: (A) plasma blank, (B) 120 ng of I.S., 50 ng of V racemate and 17 ng of NV racemate added to plasma, (C) plasma sample from a subject administered with an i.v. infusion of V racemate.

changing from manual injection to autosampler injection resulted in a loss of sensitivity. Absorption of V to the polypropylene walls of the autosampler vials and tubing could possibly explain this fact. Indeed, this hypothesis was proven. We consistently observed carry-over or wash-out peaks when pure solvent was injected by the autosampler following spiked samples. Increasing the concentration of acetonitrile in the reconstitution solvent, however, resulted in a dramatic loss of separation. Using potassium phosphate 0.01 *M*, pH 4.8, rather than the mobile phase buffer pH 6.65, improved the recovery but did not solve the problem, since there was still carry-over. However, we decided to not investigate this any further, because we were able to achieve satisfactory limits of quantitation and precision by using a water–acetonitrile (50:50, v/v) mixture rather than the mobile phase to flush the autosampler between injections.

3.2. Specificity

As indicated in Fig. 1A, no peaks from endogenous substances were detected near the retention times of the V or ANV enantiomers. Specificity regarding metabolites was assessed only for NV, which according to Hedman et al. [4] was the only metabolite interfering with V under similar chromatographic conditions. Derivatization of NV with acetic anhydride completely avoided this problem. The derivatization efficiency study showed that the amount of acetic anhydride added was sufficient to derivatize at least 445 ng/ml of NV. Based on literature

information [8], this level largely exceeds the maximum concentration of NV possibly present in plasma samples from a clinical study, when only V is administered at therapeutic doses.

The other metabolites of V are N-dealkylated (D617 and D620) or N-dealkylated and O-demethylated (PR22 and PR25). However, the latter are conjugated in plasma [9] to form hydrophilic species that would not be extracted in significant quantity during sample preparation. If the N-dealkylated metabolites are eventually extracted from plasma, they are acetylated to molecules more hydrophilic than ANV, I.S., and V, therefore they should elute faster under these experimental conditions. Hedman et al. [4] did not report any problem of interference with metabolites even after oral administration. Also in our clinical study we never notice extraneous peaks or asymmetries in the V or ANV chromatograms

3.3. Extraction efficiency

The extraction recoveries of *R*-V, *S*-V, *R*-NV and *S*-NV were 101.67 ± 9.87%, 99.53 ± 12.21%, 93.46 ± 12.0%, 89.58 ± 10.3% at the low level (10 ng/ml, $n = 5$), and 95.82 ± 5.05%, 93.48 ± 5.25%, 98.32 ± 2.27%, 93.29 ± 1.82% at the high level (100 ng/ml, $n = 5$). The extraction recovery of (+)-glaucine was 103.45 ± 10.12% ($n = 4$). It should be noted, however, that when reference samples were prepared by spiking directly 6 ml of the extraction solvent mixture, abnormal results of 150% recovery were found for V and (+)-glaucine. A possible explanation is that plasma extracts contain substances preventing adsorption of V or I.S. to the glass wall of the tube. This hypothesis was verified by extracting 1 ml of blank plasma with the organic solvent mixture. The organic phase was then spiked with the selected amount of V or (+)-glaucine after transferring it to a clean glass tube. The resulting extraction recoveries then ranged between 95 and 102%. These finding agree with those reported by Hynning et al. [8]. Silanization of the glassware did not solve this problem.

3.4. Linearity, accuracy, and precision

Linearity, accuracy and precision of the method were assessed by analyzing a calibration curve made of six different concentrations plus a blank and sample controls at four different concentrations. Each control was done in triplicate. The same procedure was repeated on three different days. The concentrations of the spiked samples for the calibration curve were 2.63, 5.26, 10.1, 25.2, 50.0, 101.5 ng/ml for single V enantiomers and 1.79, 3.55, 6.85, 17.0, 33.8, 68.6 ng/ml for single NV enantiomers. The concentrations of the quality controls were 3.20, 6.77, 37.6, 75.2 ng/ml for single V enantiomers and 2.16, 4.57, 25.4, 50.8 ng/ml for single NV enantiomers. The lowest concentrations of the quality controls represent also the limit of quantitation (LOQ).

The peak-height ratio increased linearly with the concentration over the range investigated for all four analytes, with correlation coefficients ≥ 0.995. The calibration curves were analyzed by simple and weighted (as $1/x$ and $1/x^2$, where x is the concentration) regression. The weighted regression as $1/x$ gave the most precise back-calculated values at the lowest concentrations. We used this type of regression to estimate the samples concentrations. The average slope and standard error of the slope were 2.01×10^{-2} and 7.08×10^{-4} for *R*-ANV, 1.29×10^{-2} and 1.61×10^{-4} for *S*-ANV, 1.38×10^{-2} and 1.83×10^{-2} for *R*-V, 1.19×10^{-2} and 1.43×10^{-4} for *S*-V. Intercepts were insignificantly different from zero, therefore they are not reported. Accuracy and precision were then assessed from the control standards as % relative error (% error) and as % coefficient of variation (%C.V.). The results are presented in Table 1.

4. Conclusions

The HPLC method presented is direct, simple, selective, reproducible, sensitive and linear for the simultaneous determination of V and NV enantiomers in human plasma. The method has been applied successfully to a clinical study. A single column maintained high quality separation

Table 1
Accuracy and precision

Day	Quality controls							
	3.20 ng/ml		6.77 ng/ml		37.6 ng/ml		75.2 ng/ml	
	C.V.[a] (%)	E[b] (%)	C.V. (%)	E (%)	C.V. (%)	E (%)	C.V. (%)	E (%)
R-Verapamil								
1	3.42	1.70	6.36	3.80	1.24	−1.35	1.91	2.18
2	8.70	2.66	3.16	−4.53	6.07	−3.65	6.32	4.77
3	7.28	−9.29	1.34	−8.79	2.95	−2.78	2.12	3.24
S-Verapamil								
1	4.75	−1.67	7.12	−10.3	0.21	−0.85	2.47	3.33
2	10.8	−8.73	0.99	−9.02	5.64	−4.46	6.97	3.49
3	15.2	−2.54	6.03	−10.6	1.93	−1.57	1.51	3.12
	2.16 ng/ml		4.57 ng/ml		25.4 ng/ml		50.8 ng/ml	
R-Norverapamil								
1	4.75	9.58	2.86	2.83	4.75	−1.09	4.81	0.07
2	6.11	2.81	14.5	−9.86	15.2	−11.5	6.93	−1.77
3	2.30	9.30	5.77	4.19	6.38	−5.95	9.38	3.98
S-Norverapamil								
1	13.2	−1.90	9.27	−2.88	1.22	0.28	7.95	5.16
2	7.57	5.63	8.11	8.14	15.7	−11.7	8.55	0.29
3	7.71	−5.04	3.83	−1.41	6.27	0.59	13.4	4.10

[a] C.V. (%) = 100 · (standard deviation/mean).
[b] Error (%) = 100 · (concentration found − actual concentration)/(actual concentration).

through all the validation and clinical sample analysis, for a total of approximately 1900 injections. The only disadvantage is the time required for a single chromatogram, about 40 min.

Acknowledgements

We wish to thank Mrs. Jean Ehinger of Knoll Pharmaceuticals for providing V and NV racemates, Dr. J. Longstreth of Searle for providing V and NV single enantiomers, and Dr. R.S. Pearlman and Dr. R. Balducci of the College of Pharmacy, University of Texas at Austin, for assistance in the use of MDL software.

References

[1] H. Echizen, M. Manz and M. Eichelbaum, *J. Cardiovasc. Pharmacol.*, 12 (1988) 543.
[2] M. Eichelbaum, P. Birkel, E. Grube, U. Gutgemann and A. Samogyi, *Klin. Wochenschr.*, 58 (1980) 919.
[3] G.R. McAllister and E.B. Kirsten, *Clin. Pharmacol. Ther.*, 31 (1982) 418
[4] A. Hedman, B. Angelin, A. Arvidsson, O. Beck, R. Dahlqvist, B. Nilsson, M. Olsson and K. Schenk-Gustafsson, *Clin. Pharmacol. Ther.*, 49 (1991) 256.
[5] A. Shibukawa and I.W. Wainer, *J. Chromatogr.*, 574 (1992) 85.
[6] G. Stagni, A.M.M. Shepherd, Y. Liu and W.R. Gillespie, in preparation.
[7] MACCS-II, Chemical Information Management, Users' Manuals, Molecular Design Limited, San Leandro, CA, 1992.
[8] P.A. Hynning, P. Anderson, U. Bondesson and L.O. Boreus, *Clin. Chem.*, 34 (1988) 145.
[9] M. Eichelbaum, E.G. Remberg, M. Schomerus and J. Dengler, *Drug Metabol. Dispos.*, 7 (1979) 145.