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Solid-phase extraction–high-perfomance liquid chromatography determination of verapamil and norverapamil enantiomers in urine[1]

E.B. Asafu-Adjaye*, Gerald K. Shiu

Product Quality Research Lab., DPQR, OPS, CDER, FDA, 4 Research Court, Rockville, MD 20850, USA

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Abstract

A simple, rapid, sensitive and selective method has been developed for the stereospecific determination of verapamil and its metabolite, norverapamil in urine. For sample preparation we utilized a membrane-based solid-phase extraction (SPE) disk consisting of a thin, particle-loaded membrane inserted in a plastic syringe-like barrel. The particles, which may be C_8 or C_{18} bonded phase (C_8 in this work), are embedded within a matrix of PTFE (Teflon) fibrils. Overall analyte recoveries were above 85%, even at low concentration of 3.0 ng/ml with reproducibilities (C.V. values) below 13.1%. This method of extraction has the advantage of speed and considerable reduction in solvent volumes compared to conventional SPE and solvent extraction. The separation of all the enantiomers was achieved using a single chiral stationary phase column, the cellulose-based reversed-phase, Chiralcel OD-R. Analyte concentrations of less than 3.0 ng/ml could be quantitated with C.V. values below 14%. Calibration curves were linear in the range 2.5–300 ng/ml. Intra-day and inter-day reproducibilities were 10.5–14.2% at 3 ng/ml, 4.8–9.3% at 138.5 ng/ml and 7.8–10.1% at 280 ng/ml level, respectively, for all the enantiomers.

Keywords: Verapamil; Norverapamil; Enantiomer separation

1. Introduction

Verapamil (VER, I, in Fig. 1) is a calcium channel blocker used in the management of angina, arrhythmia and hypertension. Its primary metabolite is norverapamil (NOR, II), (N-demethylated metabolite). Both VER and NOR are pharmacologically active, contain a chiral carbon and exist as enantiomers. VER is administered as a racemate although the enantiomers differ in pharmacology and pharmacokinetics.

Several methods [1–7] for the determination of the enantiomers of VER and/or NOR in biological fluids have been published. However, in many of these only one of the two compounds is assayed; when both compounds are determined simultaneously, there is usually prior achiral separation followed by the chiral assay, a process requiring two columns. Moreover, in most of the methods, the sample preparation step involves extensive extraction procedures which often make use of hazardous and toxic organic solvents such as hexane and heptane. Also, chromatographic resolution and selectivity are often poor.

*Corresponding author.

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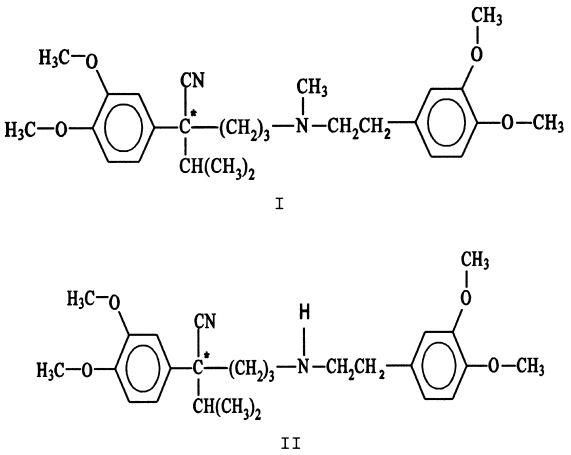

Fig. 1. Structures of verapamil (I, VER) and norverapamil (II, NOR).

Although we found the method of Shibukawa et al. [6] to be reliable for the chiral assay of VER and NOR in serum, the extraction procedure was laborious. On several occasions in our attempts, the extraction solvent, heptane, formed gels with serum and urine samples. We also observed interfering peaks from endogenous substances in the urine analysis. Thus, we decided to try solid-phase extraction (SPE) followed by the chromatographic determination on the reversed-phase CSP, Chiralcel OD-R. Chiralcel OD-R, a cellulose-tris(3,5-dimethylphenyl carbamate) stationary phase, has been shown to be capable of separating VER and NOR enantiomers when the compounds are injected separately [7]. However, in this work, we show that the separation of the enantiomers of both compounds can be achieved even when they are injected together in a mixture.

For the extraction, we used a solid-phase microextraction technique involving a disk cartridge recently introduced onto the market. The SPE cartridge used was the 3M Empore disk cartridge which consists of a thin, particle-loaded membrane inserted in a plastic syringe-like barrel. The particles, which may be C_8 or C_{18} bonded phase, (C_8 in this work), are embedded within a matrix of PTFE fibrils. The SPE procedures used with these cartridges are similar to those used with conventional SPE cartridges except for the small volumes of solvents used in the extraction.

Our objective was to develop a simple, rapid solid-phase extraction method for VER and NOR from urine, and to validate the assay for the enantiomers of these compounds using Chiralcel OD-R, a reversed-phase CSP column.

2. Experimental

2.1. Instrumentation

The instrumentation consisted of the Hewlett-Packard (Palo Alto, CA, USA) HP1090M liquid chromatograph equipped with a HP 1046A fluorescence detector. The detector was run at excitation and emission wavelengths of 230 and 312 nm, respectively. Instrument control, data acquisition and processing were done by means of the HP ChemStation software. The Chiracel OD-R CSP, 250×4.6 mm, 10 μm particle size column was obtained from Chiral Technologies (Exton, PA, USA). This column was protected with a Brownlee (Applied Biosystems, San Jose, CA, USA) C_{18} NewGuard cartridge guard column. The 3M Empore extraction disk cartridge used was the C_8, 7 mm/3 ml type and obtained from Varian Associates (Sunnyvale, CA, USA). The solid-phase extraction vacuum manifold was purchased from Supelco (Bellefonte, PA, USA).

2.2. Reagents and materials

The *RS*-(±)-verapamil·HCl, (+)-glaucine and sodium perchlorate were purchased from Sigma Chemicals (St. Louis, MO, USA). *RS*-(±)-norverapamil, *R*-(+)- and *S*-(−)-verapamil were obtained from RBI (Natick, MA, USA). All the solvents used were HPLC grade, and were obtained from either Sigma or Burdick and Jackson (Muskegon, MI, USA). All reagents were used as received.

The stock and working solutions of the analytes were all prepared in distilled deionized water. Stock solution of the internal standard, (+)-glaucine, was prepared in 50% methanol in water and subsequently diluted with deionized water to yield the 400 ng/ml working standard solution.

2.3. Standards

Calibration and control standards were prepared by spiking 0.5 ml blank urine samples with the appropriate aliquot of 1 mg/ml or 20 mg/ml racemic mixture of VER and NOR working standard solution. The concentrations of the calibration standards ranged from 5 to 600 ng/ml while the three control standards contained 6, 277 and 560 ng/ml each of RS-VER and NOR. These samples were spiked with 50 ml (+)-glaucine just before extraction.

2.4. Sample preparation

To each 0.5 ml urine sample in a glass tube was added 50 ml of the internal standard followed by 0.5 ml of 0.03 *M* phosphate buffer, pH 10 and mixed thoroughly. To condition the disk cartridges on the vacuum manifold, 500 ml of methanol was drawn through at a pressure of 5–7 inches mercury (in Hg). This was repeated making sure the disk did not dry out. Similarly, 2×500 μl of distilled water was drawn through the disk, and without letting the disk dry, the sample was loaded onto it. All of the buffered sample mixture (≈1.05 ml) was drawn through the membrane at a pressure of 7–10 in Hg. When all the sample had gone through, full vacuum was applied for a few seconds. The membrane was then washed twice with 500 ml of deionized water followed by 2×500 ml of 30% acetonitrile in water. The analytes were eluted with 2×500 ml of methanol into clean glass tubes at a pressure of 7–10 in Hg. Full pressure was applied in the end to elute the last traces of the analytes in the disk membrane. The samples were then vacuum-dried at 60°C and reconstituted in 250 ml of the mobile phase and 100 ml injected. The mobile phase consisted of 40% acetonitrile in 0.2 *M* sodium perchlorate. Flow-rate was set at 0.8 ml/min. The fluorescence detector wavelengths were set at 230 nm excitation and 312 nm emission.

3. Results and discussion

The choice of the previously mentioned chromatographic conditions was established from preliminary investigations. These conditions gave the optimum chromatographic separation; i.e. parameters such as separation factor, α, resolution, R, and peak symmetry as well as reproducibility of k' and peak asymmetry were much better than under other conditions investigated. Therefore, these were the conditions that were used throughout the work. Although we did not investigate other metabolites of VER (e.g., D617, D620, PR22, PR25, etc.) virtually no interfering peaks were observed. We think that these metabolites, being more polar than VER and NOR, may have been eluted from the disk cartridge with the repeated 30% acetonitrile–water washes. In addition, any remaining traces injected onto the column with VER and NOR may have come out of the column with the solvent front.

Figs. 2–4 show typical chromatograms for urine spiked with racemic VER and NOR, blank urine and the urine of a healthy adult subject 2–4 h after dosing with 80 mg immediate-release (IR) dose of *RS*-VER, respectively. All the peaks were baseline-resolved, with very good selectivity for the analytes. The elution order of the enantiomers was determined by chromatographing pure enantiomers of VER on the Chiralcel OD-R column. The retention times for the enantiomer peaks were as follows, with the C.V. values in parentheses: *R*-NOR – 14.5 min (1.7%), (+)-Glaucine – 16.5 (2.3%), *S*-NOR – 18.7 (2.7%), *R*-VER – 21.8 (2.5%) and *S*-VER – 24.2 (3.6%). The order was the exact opposite of that in a normal phase system using Chiralpak-AD. The chiralcel OD-R column was very stable under the conditions of the analysis such that only one Chiralcel OD-R column was used throughout this study, averaging hundreds of injections. We also observed no significant deterioration in the efficiency of the column.

Tables 1 and 2 summarize the results from recovery and validation studies, respectively, while Table 3 shows the urine concentrations of VER and NOR enantiomers in the adult subject 2–4 h after dosing with 80 mg immediate-release (IR) dose of *RS*-VER.

The extraction recovery studies were conducted at three (low, medium and high) concentration levels by spiking blank urine samples with appropriate aliquots of a mixture of racemic verapamil and norverapamil to obtain 3, 138.5 and 280 ng/ml of each enantiomer, respectively. The results indicated good recoveries (>80%) of all the analytes at all

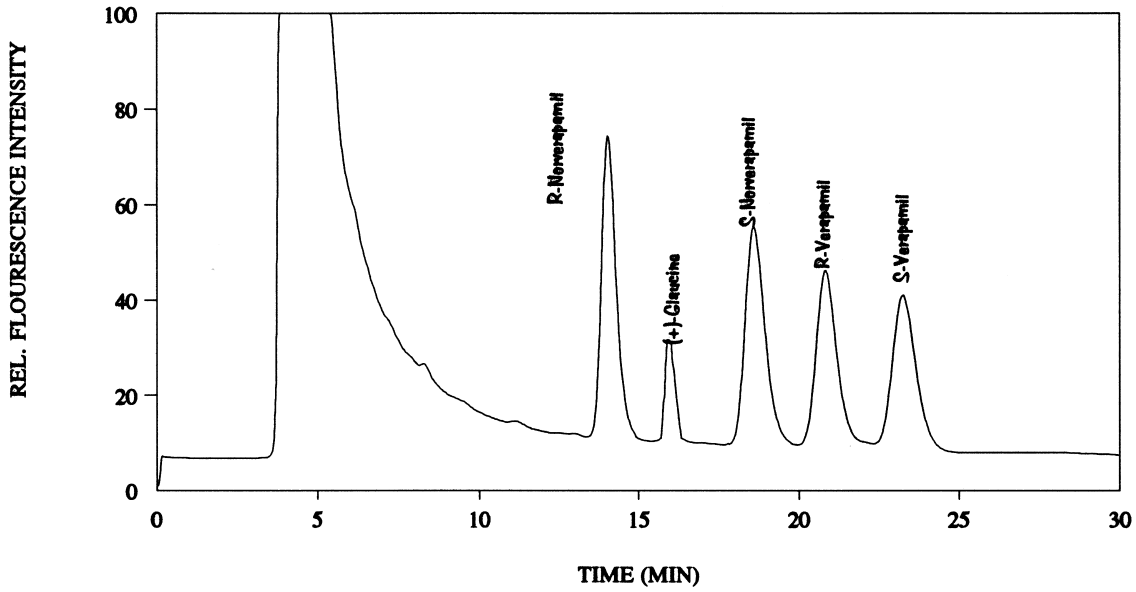

Fig. 2. Chromatogram of spiked urine extract containing VER and NOR, 200 ng/ml of each enantiomer and (+)-glaucine (internal standard). Conditions: same as in Table 1.

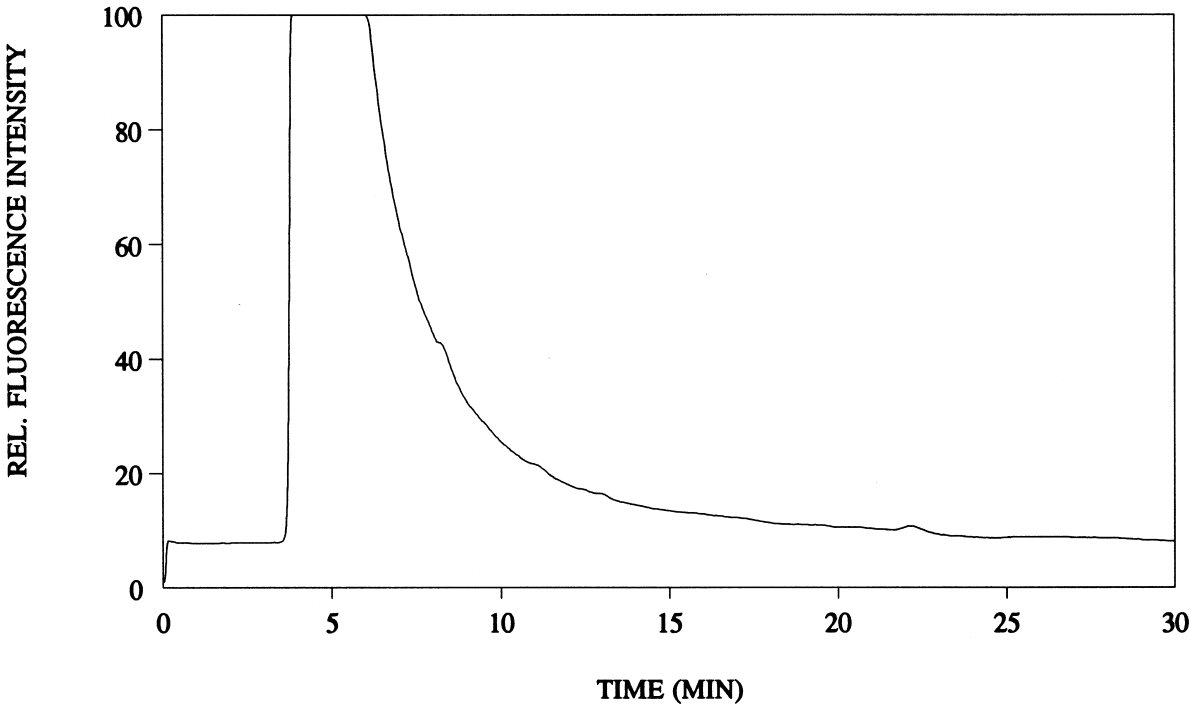

Fig. 3. Chromatogram of blank urine extract. Conditions: same as in Table 1.

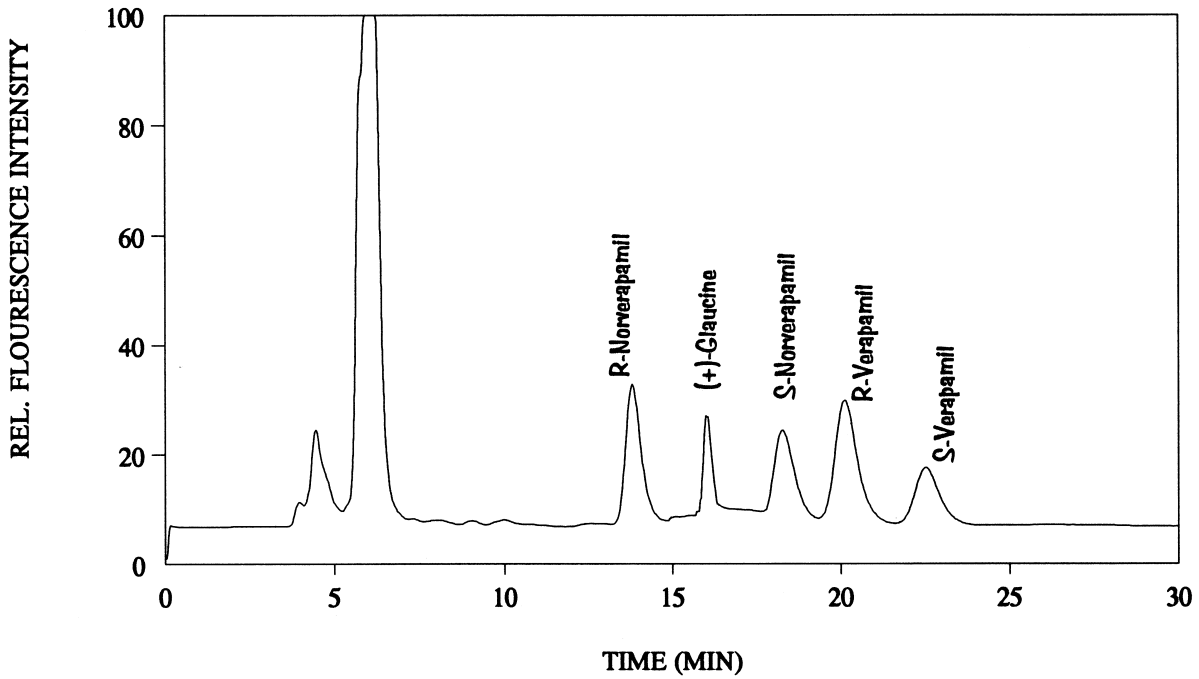

Fig. 4. Chromatogram of the 2–4 h urine extract of a healthy adult subject after oral administration of 80 mg IR dose of *RS*-verapamil. Conditions: same as in Table 1.

Table 1
Recoveries of verapamil and norverapamil enantiomers in urine

Sample	Amount added (ng/ml)	Average recovery (%) (n=6)	C.V. (%) (n=6)
R-Verapamil	3.0	106.8	10.8
	138.5	98.0	2.1
	280.0	99.9	10.8
S-Verapamil	3.0	99.1	8.3
	138.5	94.2	3.9
	280.0	101.3	10.3
R-Norverapamil	3.0	101.4	12.6
	138.5	97.9	4.4
	280.0	89.2	12.7
S-Norverapamil	3.0	100.8	10.8
	138.5	99.3	3.9
	280.0	90.1	13.1
(+)-Glaucine	40.0	86.5	12.8

Conditions: SPE cartridge – C_8 3M Empore disk cartridge, 7 mm/3 ml; HPLC column – Chiralcel OD-R, 250×4.6 mm, 10 μm; Mobile phase – 40% acetonitrile in 0.2 *M* sodium perchlorate at 0.8 ml/min; Detection – fluorescence detector, Ex=230 nm and Em=312 nm.

Table 2
Intra-day and inter-day reproducibilities of verapamil and norverapamil enantiomers in urine

Compound	Spiked concentration (ng/ml)	Intra-day (n=6)		Inter-day (n=6)	
		Mean determined concentration (ng/ml)	C.V. (%)	Mean determined concentration (ng/ml)	C.V. (%)
At low concentration					
R-Verapamil	3.00	3.04	11.8	2.82	12.6
S-Verapamil	3.00	2.96	12.9	2.97	13.1
R-Norverapamil	3.00	3.03	9.1	3.03	10.5
S-Norverapamil	3.00	3.03	11.3	3.18	14.2
At medium concentration					
R-Verapamil	138.5	134.03	5.2	140.2	8.5
S-Verapamil	138.5	132.15	5.9	141.3	9.3
R-Norverapamil	138.5	136.02	3.1	138.6	4.8
S-Norverapamil	138.5	136.30	3.3	139.1	6.5
At high concentration					
R-Verapamil	280.0	271.10	6.2	283.5	7.8
S-Verapamil	280.0	273.19	7.4	269.8	9.5
R-Norverapamil	280.0	279.23	7.4	269.8	10.1
S-Norverapamil	280.0	284.86	6.3	279.8	8.9

Conditions: same as in Table 1.

levels. Reproducibilities, (n=6) as measured by the coefficients of variation (C.V.), were 8.3–12.6% at the low level, 2.1–4.4% at medium and 10.3–13.1% at the high level for all four enantiomers. The intra-day validation studies yielded C.V. values of 9.1–12.9% at a low analyte level of 3 ng/ml, 3.1–5.9% at the 138.5 ng/ml level, and 5.4–7.4% at the higher level of 280 ng/ml for all the enantiomers. Similarly, inter-day C.V. values ranged from 10.5–14.2% at the lower level, 4.8–9.3% at the medium level and 7.8–10.1% at the higher level, respectively. The relative error associated with the determined concentration of each enantiomer compared with the spiked concentration was 1.3% or less for the low concentration, 4.6% or less for the medium concentration and 3.2% or less for the high concentration level, respectively. These figures compare favorably with those of Shibukawa et al. [6] for VER and NOR enantiomers in plasma.

4. Conclusion

In this work, we have demonstrated the use of the solid-phase microextraction technique combined with reversed-phase chiral stationary phase liquid chromatography to determine the enantiomers of verapamil and its metabolite, norverapamil, in urine. Although

Table 3
Concentrations of verapamil and norverapamil enantiomers in urine of a subject after oral administration of 80 mg IR dose of *RS*-verapamil

Sample	Concentration determined (ng/ml)[a]			
	R-Verapamil	*S*-Verapamil	*R*-Norverapamil	*S*-Norverapamil
0–2 h urine	781.8 (5.4)	693.8 (4.5)	392.9 (7.3)	367.8 (10.9)
2–4 h urine	158.3 (3.2)	81.2 (6.6)	107.8 (5.8)	99.8 (8.1)
4–8 h urine	59.6 (4.1)	27.6 (5.9)	101.6 (9.1)	95.8 (5.8)
8–12 h urine	38.9 (6.1)	17.9 (10.1)	41.5 (8.7)	31.3 (9.2)

Conditions: same as in Table 1.
Mean values (n=6), with C.V. values in parentheses.

this was demonstrated in human urine, we can envision its application to serum and other biological fluids with little or no modification; there have been several reports [8] of the use of the disk cartridge for the extraction of a variety of drugs from plasma and serum with little or no interference from proteins. The solid-phase microextraction with the disk cartridge yielded excellent recoveries for VER and NOR enantiomers. While the method was simple, rapid and reproducible, it involved the use of reduced volumes of solvents compared to conventional solid-phase or solvent–solvent extractions. The extraction procedure can be easily automated as has been happening lately for conventional SPEs.

The assay on the Chiralcel OD-R reversed-phase chiral stationary phase column, was specific and sensitive for the enantiomers of VER and NOR with a LOQ of approximately 3 ng/ml. The signal response was linear from 2.5–300 ng/ml and had excellent accuracy and reproducibility. In addition, the reversed-phase nature of the column made it possible to avoid the use of toxic nonpolar solvents such as hexane, which are not always easy to work with as a result of their volatility. Besides, this method utilizes only one column with a simple solvent system to achieve the stereoselective separation of *R*- and *S*-VER from *R*- and *S*-NOR. It could easily be used for routine analysis in a clinical setting, or for pharmacokinetic studies of the enantiomers of VER and NOR.

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