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Simultaneous determination of verapamil and norverapamil in biological samples by high-performance liquid chromatography using ultraviolet detection

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Abstract

In this paper we develop an high-performance liquid chromatographic method with ultraviolet detection for the determination of verapamil and its primary metabolite norverapamil in biological samples. Both compounds, as well as the internal standard, imipramine, were extracted from alkalinised blood, with *n*-hexane–isobutyl alcohol, back-extracted into 0.01 *M* phosphoric acid and determined using a reversed-phase column and ultraviolet monitoring at 210 nm. The average coefficient of variation obtained over the concentration range of 1–1000 ng/ml is about 3%. The detection limit is below 5 ng/ml for both compounds, and extraction recoveries close to 80%. The method was applied to a pharmacokinetic study of the drug and its active metabolite and used to analyse blood samples from verapamil treated rabbits.

Keywords: Verapamil; Norverapamil

1. Introduction

Verapamil hydrochloride [DL-2-(3,4-dimethoxyphenyl)-2-isopropyl-5-(*N*-methyl-*N*-β(3,4-dimethoxyphenyl)ethylamino)valeronitrile] (Fig. 1), a synthetic papaverine derivative, is a well-known calcium channel blocker which has antianginal, antihypertensive and antiarrhythmic properties [1]. This compound is extensively metabolised by *N*-demethylation and *N*-dealkylation to several metabolites. The *N*-demethylated metabolite of verapamil (norverapamil) (Fig. 1) is pharmacologically active. Moreover, it could accumulate in the body at concentrations equal to or even greater than those of the parent drug [2–7].

Verapamil is commercially used as a racemic mixture of equal amounts of two optical isomers, (+)-(*R*)-verapamil and (−)-(*S*)-verapamil. The optical isomers differ considerably in their pharmacological potency with (−)-(*S*)-verapamil being 10–20 times more potent than the (+)-(*R*)-verapamil in terms of negative dromotropic effect on AV conduction in man [8] and in dogs [9]. Moreover, the first-pass metabolism and plasma protein binding of racemic verapamil is stereoselective in man [10]. In addition, it has been showed that the pharmacokinetics of the verapamil enantiomers differ from each other after single intravenous and oral

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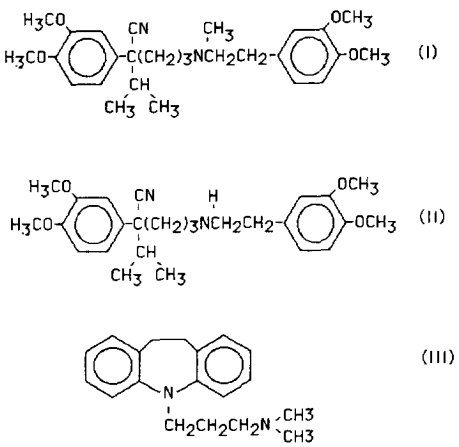

Fig. 1. Chemical structure of (I) verapamil, (II) norverapamil and (III) imipramine.

doses in man. In this sense, several HPLC methods with fluorescence detection have been developed to separate the enantiomers of verapamil [11–17] and they have been successfully applied for pharmacokinetic and clinical studies [18–20]. In these methods, a large time of analysis and/or elaborate chromatographic systems are necessaries in order to elucidate between the two enantiomers. In contrast to studies in dogs and man, the pharmacokinetics of the verapamil enantiomers does not appear to differ in other animal species, such as rabbits [21]. Thus, the development of a simple HPLC method which permits a simple detection of the racemic mixture of verapamil and its active metabolite norverapamil could be interesting.

Moreover, we are particularly interested in the use of UV detection, because other calcium channel blockers (CCB), such as diltiazem, nifedipine and nitrendipine, must use the same detection system (these compounds do not present luminescence). This fact could be interesting when a simultaneous pharmacokinetic analysis, monitoring or clinical studies could be carried out. Unfortunately, the two HPLC methods described in the literature for verapamil and norverapamil [22,23], which use UV detection are not suitable for biological studies for a variety of reasons (e.g., prolonged time elution and analysis which biological samples studies have not been used).

Our main objective has been to develop a simple, time and money-saving HPLC method which uses UV detection for the simultaneous determination of verapamil and norverapamil in biological samples, in order to carry out pharmacokinetic studies in animal species where a stereoselective detection is not necessary. Finally, we check out this method by describing the pharmacokinetics of verapamil after intravenous administration in the rabbit.

2. Experimental

2.1. Chemicals and reagents

Verapamil and norverapamil hydrochloride (Fig. 1) were supplied by Knoll Laboratories. Stock solutions containing 100 mg/ml (free base) of each of the products in water were prepared and stored at −20°C. The internal standard imipramine (10,11-dihydro-*N*,*N*-dimethyl-5H-dibenz[*b*,*f*]azepine-5-propanamine hydrochloride (Fig. 1) was obtained from Sigma and was used as an aqueous solution (3 mg/ml), that was prepared daily by dilution from a stock solution containing 100 mg/ml imipramine (free base) in water, that was itself made weekly and stored at 4°C. Methanol, acetonitrile, *n*-hexane, isobutyl alcohol and phosphoric acid were all HPLC-grade and obtained from Scharlau Reagent. Sodium hydroxide was used as an aqueous solution in water (2 *M*). Ammonium acetate and triethylamine were both analytical reagent grades. Glass distilled water was purified through a Milli-Q system.

2.2. Extraction procedure

A 2 ml volume of blood (unknown sample, or drug-free blood, or blood standard containing known amounts of the drugs) and 2 ml of cold water (in order to hemolyse blood samples) were transferred to 25 ml glass centrifuge tubes and spiked with 0.2 ml of the internal standard. The samples were alkalinised by addition of 0.1 ml of 2 *M* NaOH. Following addition of 10 ml of organic phase (a mixture of *n*-hexane–isobutyl alcohol, 98:2, v/v), the tubes were shaken horizontally at 200 oscilations/min on a Selecta Rotavit agitator for 30 min and then centrifuged in a Kubota 2000 apparatus at 600 *g* for 5 min. Samples were then frozen at −20°C and the upper organic phase poured into a new tube con-

taining 0.15 ml of 0.01 *M* phosphoric acid. The tube was vigorously shaken on a vortex mixer for 1 min and centrifuged at 600 *g* for 5 min.

The samples thus treated were frozen at −20°C, the organic phase was discarded and a 0.075-ml aliquot the aqueous phase was injected into a high-performance liquid chromatograph (HPLC).

2.3. HPLC procedure

A Waters 501 HPLC pump and a Waters M 717 autosampler were employed. Separations were performed on a NovaPak C_{18} (150×3.9 mm I.D.) reversed-phase column packed with 4-μm particles. A NovaPak C_{18} HPLC pre-column from Guard-Pak inserts was used between the injector and the analytical column to effectively minimise accumulation of particulate matter on the analytical column. Both columns were used at room temperature (approx. 22°C).

The mobile phase consisted of a mixture of methanol–0.04 *M* ammonium acetate–acetonitrile–triethylamine (2:2:1:0.04, v/v). The pH was adjusted to 7.10 by addition of 0.1 *M* acetic acid. The mobile phase was filtered through a 0.45 μm Lida filter, prior to use. The HPLC system was operated isocratically at room temperature. The effluent flow-rate was 1.2 ml/min. The eluate was continuously monitored for absorbance at 210 nm using a Waters 484 tunable absorbance selectable-wavelength UV–Vis detector. Area integrations, peak height measurements, calculations and plotting of the chromatograms were all carried out by a Acer Model Acros 486 DX computing integrator with a PC Integrator pack program.

2.4. Calibration procedure

Calibration curves were constructed by transferring appropriate volumes of stock solutions of verapamil and norverapamil to glass centrifuge tubes containing hemolysed drug-free blood (4 ml) in appropriate amounts (10–100 μl) to give final concentrations in the range of 1–1000 ng/ml. These calibration samples were then taken through the extraction procedure described in Sections 2.2 and 2.3.

The calibration curve was characterised by its regression coefficient, slope and intercept, and used to determine the analyte concentrations in the samples and the detection limits [24]. Final sample concentrations were calculated by determining the peak area ratios of verapamil and norverapamil to internal standard, and interpolating them in the standard curves obtained for the calibration samples.

2.5. Recovery, sensitivity, accuracy and precision

Recoveries were determined by extracting ten samples containing 50, 200 and 400 ng/ml verapamil and norverapamil as described in Sections 2.2 and 2.3, followed by the addition of further verapamil and norverapamil to five of them. All samples was analyzed and the ratio of verapamil and norverapamil to internal standard for the two sets of samples was then compared.

The inter- and intra-day accuracy and precision of the method were determined by adding variable amounts (25, 50, 100, 200 and 500 ng/ml) of both analytes to drug-free blood and measuring ten samples at each concentration.

2.6. Application of the method

Three New Zealand white rabbits (body weights up 4 to 4.5 kg) were used in a pharmacokinetic study. Verapamil hydrochloride (Manidon, Knoll) was administered intravenously as a bolus dose of 1 mg/kg in the ear marginal vein. Serial blood samples (2 ml) were drawn into tubes containing cold water (2 ml) at 5, 15, 30, 60, 90, 120, 180, 240, 380 and 480 min after administration. Samples were stored at −20°C until analysis.

3. Results and discussion

3.1. Analytical results

The extraction recoveries of verapamil and norverapamil from the blood samples obtained by the proposed method, are given in Table 1. Both extraction recoveries were independent of the analyte concentration over the range tested, thereby sug-

Table 1
Extraction recoveries for verapamil and noverapamil from biological samples

Concentration	Recovery (mean±S.D., $n=10$) (%)	
(ng/ml)	Verapamil	Norverapamil
50	72.5±7.2	77.1±4.4
200	84.3±8.8	78.0±5.4
400	80.9±8.0	76.3±3.8
%Mean±S.D.	79.2±6.1	77.8±5.9

gesting acceptable efficiency for the extraction procedure.

The extractability of both compounds from blood was tested in recovery experiments using various solvent extraction systems, different *n*-hexane–isobutyl alcohol mixtures and different ratios of extraction solvent to sample volume. The optimal ratio was 1:5 for blood sample–organic mixtures and 4:0.25 for mixture–acid solvent. The maximum extraction recovery was obtained by using *n*-hexane–isobutyl alcohol (98:2, v/v) (over 84% and 78% for verapamil and norverapamil, respectively). Both values are higher than previously reported recoveries (approx. 60%) [25]. Back-extraction into 0.01 *M* phosphoric acid greatly decreased the interference from plasma constituents. Phosphoric acid concentrations higher than 0.01 *M* do not improve the extraction procedure and could decrease the lifespan of the chromatographic column.

Retention times under the described chromatographic conditions were 3.6, 4.9 and 7.3 min for norverapamil, verapamil and imipramine, respectively. As can be seen in Fig. 2, no interfering peaks appeared at these retention times, and all compounds eluted as separate symmetrical peaks. No interference from endogenous blood constituents was observed. A C_{18} reversed-phase column, as well as methanol–0.04 *M* ammonium acetate–acetonitrile and triethylamine as the mobile phase, effectively afforded separation of the analytes in about 7 min. Other reversed-phase packings (C_1, C_2, C_8) have been reported for separation of the analytes, but separation was worse than when using a C_{18}, because the retention times were too long or plasma peaks overlapped with each other [25–28].

The presence of ammonium acetate was essential for complete separation of the analytes. In fact, its

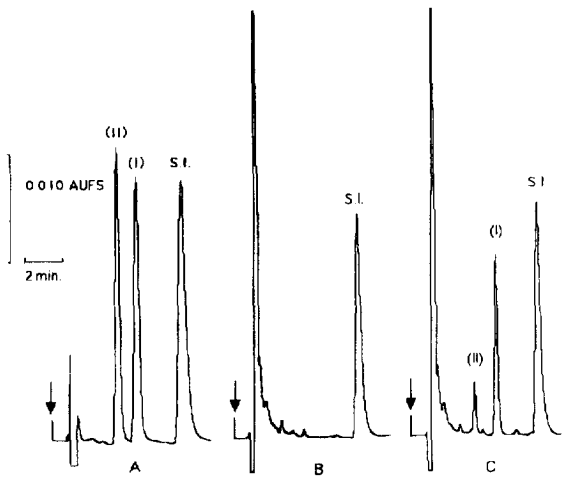

Fig. 2. Typical chromatograms for verapamil (I), norverapamil (II) and I.S. (A). Standard peaks obtained by injecting 300 ng I, II and 800 ng I.S.. (B) Drug-free blood samples. (C) Drug-free blood to which was added 250 ng I, 75 ng II and 800 ng I.S..

replacement with water or an acetate buffer led to a loss of resolution. Nevertheless, the ammonium acetate concentration is not critical if it is equal to or above, 0.04 *M*. Columns need not be longer than 150 mm, unless other minor metabolites are to be resolved as well. The pH of the mobile phase was very important in order to maintain adequate separation of the analytes. The chromatographic separation of verapamil, norverapamil and the internal standard was very sensitive to the pH of the mobile phase; in addition, careful adjustment of the pH enabled selective separation by ionisation of specific compounds. The optimum pH was 7.10. The triethylamine content reduced retention time of verapamil and its metabolite norverapamil and greatly improved peak shapes at concentration up to 0.04%, beyond which there were no further improvements. Imipramine, despite its structure (similar to verapamil) was chosen as the internal standard, because the physicochemical properties of the two are close. Moreover, imipramine could be extracted from plasma under the extraction conditions described in Sections 2.2 and 2.3. Finally, imipramine was found to provide the best results from amongst all the internal standards tested.

The verapamil and norverapamil standard curves were linear over the range from 1 to 1000 ng/ml

Table 2
Accuracy and precision of the HPLC method for the determination of verapamil

Theoretical verapamil blood concentration (ng/ml)	Observed verapamil blood concentration (mean±S.D., $n=10$) (ng/ml)	C.V. (%)	Accuracy (%)
Intra-day			
25	25.3±0.5	2.0	101.2
50	47.2±1.3	2.8	94.4
100	96.4±4.0	2.4	96.4
200	206.6±3.8	1.8	103.3
500	503.1±9.0	1.8	100.6
Inter-day			
25	24.8±0.6	2.4	99.2
50	48.7±1.1	2.3	97.4
100	94.7±2.7	2.9	94.7
200	201.2±3.8	1.9	100.6
500	493.1±7.9	1.6	98.6

($y=0.2458x-0.622$ and $y=0.3427x+0.471$, $r^2=$ 0,999 for both drugs, $n=10$). Furthermore, the detection limit was 5 ng/ml for verapamil and 3 ng/ml for norverapamil. This linearity range and its detection limits enable us to use this method for pharmacokinetic studies and therapeutic monitoring. What is more, this method supposes a marked improvement in comparison with those described by other authors [22,25,27,29].

The coefficient of variation was less than 3% for both compounds. The accuracy and precision results for the proposed method are summarized in Tables 2 and 3.

As a result of our experiments, we have developed a chromatographic method using UV detection for simultaneous determination of verapamil and norverapamil with internal standard in biological samples. This analytical method supposes a significant improvement on the previous methods in some respects such as linearity range (1–1000 ng/ml), retention times (which are less than 10 min), good sensitivity (detection limit lower than 5 ng/ml) and adequate accuracy and precision. Moreover, the different biological samples analysed do not present any interfering peaks with the analytes. For all these reasons, our method may be suitable for the determination of these compounds in biological matrices and its application to pharmacokinetic studies.

3.2. Pharmacokinetic results

The measurement of blood levels of verapamil and norverapamil in the rabbit after intravenous administration confirmed the utility of the analytical methodology.

Fig. 3 shows the mean variations of the plasma concentrations over time for all the subjects studied. Relevant pharmacokinetic parameters (elimination

Table 3
Accuracy and precision of the HPLC method for the determination of norverapamil

Theoretical norverapamil blood concentration (ng/ml)	Observed norverapamil blood concentration (mean±S.D., $n=10$) (ng/ml)	C.V. (%)	Accuracy (%)
Intra-day			
25	23.6±0.6	2.5	94.4
50	46.8±1.4	3.0	93.6
100	94.4±2.0	2.1	94.4
200	213.7±4.0	1.9	106.8
500	505.9±5.7	1.1	101.2
Inter-day			
25	26.1±0.7	2.7	104.6
50	51.8±0.4	0.8	103.6
100	98.4±0.8	0.8	98.4
200	202.0±2.5	1.2	101.0
500	506.5±3.6	0.7	101.3

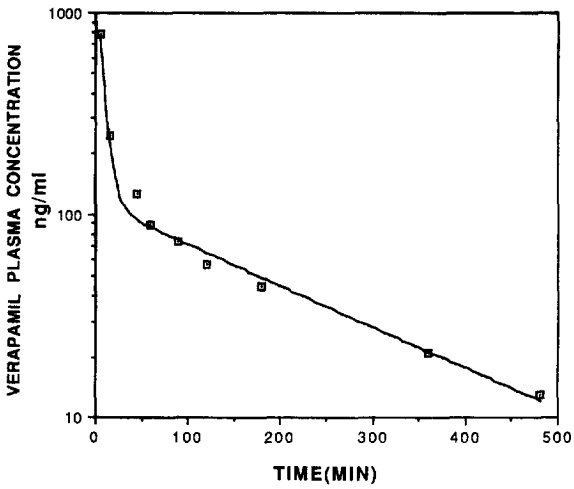

Fig. 3. Semilogarithmic plot of mean concentration vs. time of verapamil in plasma obtained after administration of a dose 1 mg/kg body weight. The open squares and the solid line represent, experimental values and the best fit to the exponential equation described above, respectively.

half-life, body clearance, etc.) were calculated by classical equations used in compartmental analysis [30] and agree precisely with those obtained by Giacomini et al. [21].

4. Conclusions

We have developed a specific, sensitive, simple, rapid and reproducible HPLC method for the determination of verapamil and one of its main metabolite (norverapamil) in the blood. The assay involves a simple extraction procedure and separation by reversed-phase HPLC using internal standard as an aid to quantification, and UV detection is employed as a new detection method. This method has been applied in order to determine verapamil and norverapamil in the blood of the rabbit, after intravenous administration of verapamil, with satisfactory accuracy and precision. In addition, the resulting run time is suitable for processing numerous samples on a daily basis.

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