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Short communication

A validated method for the determination of verapamil and norverapamil in human plasma

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Abstract

The aim of the study was to develop a simple and sensitive analytical method to determine verapamil (**V**) and its metabolite norverapamil (**N**) in human plasma with use of an HPLC isocratic system with fluoresence detection, following fast extraction of the investigated compounds. Extraction recovery was 92.12% and 89.58% for **V** and **N**, respectively. Internal standard in HPLC was propranolol. Its recovery was 82.50% on the average. Limit of detection was 0.924 ng/ml and limit of determination was 3.080 ng/ml for **V,** what corresponds concentration in plasma 1.232 ng/ml. For **N** limit of detection was 0.030 ng/ml and limit of determination was 1.001 ng/ml what corresponds 0.4 ng/ml in plasma.

Parameters of validation prove that precision of the presented method is very good. The method is fast and one chromatogram separation lasts about 8 minutes. 30–40 manual (without autosampler) analyses per day were done. It was used successfully in pharmacokinetic and bioavailability studies of verapamil administration in drug formulations alternative to tablets: buccal and flotation ones.

Keywords: Verapamil; Norverapamil; Human plasma; Fast extraction; HPLC; Fluorescence detection; Validated method

1. Introduction

Verapamil hydrochloride (**V**) is a to calcium channel antagonist. It is used in the treatment of hypertension and angina pectoris. In medical practice it is mostly used in a conventional tablet form in a minimal dose of 40 mg and a maximal dose of 180 mg and in slow release form in doses of 120 and 240 mg, respectively. Only 10–20% out of the 90% of the dose absorbed from the digestive tract penetrates to the circulatory system in an unchanged form [1]. This is due to a marked first pass effect, mostly in liver. **V** undergoes presystemic elimination in the liver before distribution in the circulation and reaching the site of pharmacological of ac-

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tion [2]. In drug formulations currently **V** is present in racemate mixtures. Enantiomers R(+) and S(−) possess different pharmacokinetic properties [3]. S(−) verapamil elicits a 20-, 15- and fivefold stronger dromotropic (−), inotropic (−) and chronotropic (−) effect, respectively, than the R(+) enantiomer. Depending on the formulation, the therapeutic concentration of **V** in human plasma ranges from 20 to 500 ng/ml [4]. Biological half-time of **V** is 3–7 h [5].

V is metabolized mainly in human liver to six (according to some authors to twelve) metabolites, which are excreted in the urine [6]. Only one metabolite, norverapamil (**N**), has pharmacological activity. It shows 20% efficacy with **V** in regard to the vasodilatant effect, but is devoid of antiarhytmic activity [7].

To quantitate **V** and **N,** the concentrations in plasma are at present measured by the following analytical methods: high-performance liquid chromatography (HPLC) [5,8–12], gas chromatography (GC) [13,14] and a hyphenated technique: gas chromatography–mass spectrometry (GC–MS) [15]. The separation of enantiomers of **V** and **N** is realized mostly with use of a GC and HPLC method with chiral stationary phases [16–18] or by capillary electrophoresis [19]. In HPLC methods a fluorescence rather than a UV detector is used. Most of these methods are of a high sensitivity, but at the same time, they employ complicated extraction procedures of **V**, **N** and the internal standard.

The purpose of this work was to elaborate a simple and sensitive analytical method to determine the concentration of **V** and its metabolite **N** in human plasma with the use of an HPLC isocratic system and fluorescence detection following a fast extraction of the investigated analytes. The method was applied in pharmacokinetic studies of **V** and **N** after its administration to healthy volunteers in a buccal drug formulation comprising 20.0 mg of verapamil, as well as in flotation pellets comprising 40.0 mg of the individual drug . The new drug formulations mentioned were designed to provide a more effective **V** dosing in comparison to the conventional 40.0 mg verapamil tablets [20,21].

2. Materials and methods

Determination of **V** and **N** was performed by HPLC with fluorescence detection. Verapamil was obtained from Sigma Chemical Co. (St. Louis, USA) and norverapamil was obtained from Knoll AG (Ludwigshafen, Germany). Propranolol hydrochloride was from Rosemont Pharmaceuticals (Leeds, UK).

Standard solutions of **V** and **N** (both hydrochlorides) and of propranolol hydrochloride were prepared by dissolving appropriate amounts of the substance in the mobile phase. The mobile phase was acetonitrile/0.05 M phosphate buffer pH 3 40/60 v/v. The flow rate of the mobile phase was 1.0 ml/min.

Acetonitrile (HPLC grade) was from J.T. Baker (Phillipsburg, USA). Water was prepared with an Elix 3 System (Millipore Corporation, Bedford, MA, USA). The phosphate buffer (0.05 M, and pH 3.0) was prepared by dissolving the appropriate quantity of potassium dihydrogen phosphate (J.T. Baker Phillipsburg, USA) in pure water, and adjusting the pH with phosphoric acid (Fluka Chemie AG, Buchs, Switzerland).The pH of the buffer was measured at 21°C before addition of the organic modifier. The measurements were carried out with an HI 9017 pH-meter made by Hanna Instruments, Bedfordshire, England.

Chromatographic measurements were made with an HPLC system Varian 9050 (Varian, Chromatography Systems, Walnut Creek, USA) consisting of a quaternary pump model 9012, a manual sampler with 50.0 μl loop model 7125 (Rheodyne Cotati, CA, USA) and a fluorescence detector model 7125 (Varian, Chromatography Systems, Walnut Creek, USA). Chromatographic data collection was carried out by means of Chomik software (Boltzmann Morchines, Warsaw, Poland) for process control and data handling. The separations were performed at room temperature on a Supelcosil LC-8-DB column, 150 × 4.6 mm I.D., particle size 5 μm (Supelco Co., Bellofonte, CA). Precolumn was LC-8-DB, 20.0 × 4.6 mm I.D., particle size 5 μm obtained from the same supplier.

Spectrofluorimetric detection was at 204 nm (excitation) and 314 nm (emission).

The extraction from human plasma was carried out as follows. To 0.5 ml of plasma 50.0 ng of internal standard (30 μl of a standard solution in the mobile phase) and 2.0 ml of acetonitrile were added. The extraction was carried out over 10 min by shaking in glass test-tubes with teflon caps Chromacol (Chromacol LTD, Trumbull, USA). The organic layer was separated by centrifugation at 5000 rpm for 10 min and then transferred into a Pyrex conical tube (Medlab, Warsaw, Poland). Organic solvent was evaporated at room temperature under nitrogen. Dry residue was dissolved in 100.0 μl of the mobile phase and a 50.0 μl sample was injected into the HPLC system.

The method was subjected to a validation procedure. The following results were obtained:

2.1. Calibration and linearity

Linear regression analysis using a least-square fit was performed. The calibration curve was obtained from standard samples of the following concentrations: 5.0, 25.0, 50.0, 125.0 and 250.0 ng/ml. Means of six independent measurements were taken into consideration. Calibration standards were injected directly onto the HPLC column (three times each).

2.2. Recovery

Recovery was calculated by comparing peak areas of standards after extraction with the peak areas of standard solutions which corresponded to a 100% recovery.

2.3. Precision

Precision of the method was calculated on the basis of inter-assay reproducibility (data from calibration) and intra-assay reproducibility. Data determination on six different days were considered.

2.4. Accuracy

Data derived from intra-assay reproducibility studies were used.

2.5. Limit of detection and limit of determination

Limit of detection was calculated as three standard deviations (SD) and limit of determination as ten SD observed after injection of standards at concentration 1 and 4 ng/ml for **V** and **N**, respectively.

3. Results and discussion

Calibration curves were derived from three injections of five concentrations of **V** and **N** (5.0, 25.0, 50.0, 125.0 and 250.0 ng/ml) plus 50.0 ng of the internal standard propranolol hydrochloride (in each calibration sample). The following regression equations were obtained:for verapamil:

$$y = 0.412x + (-0.16) \quad (1)$$

for norverapamil:

$$y = 0.478x + (-0.025) \quad (2)$$

Correlation coefficient, r, for Eq. (1) is 0.999 and for Eq. (2) r equals 0.998.

Recovery was 92.12 (variability index, CV = 3.028%) and 89.58% (CV = 1.620%) for **V** and **N**, respectively. Stability in the whole range of the calibration curve was found.

Internal standard (propranolol hydrochloride) was extracted with on average a 82.50% yield (CV = 3.720% for $n = 34$).

Detailed information concerning the precision of the method is given in Tables 1 and 2.

The limits of detection and limit of determination for **V** and **N** were determined by using 1.0 ng of standard **V** and 4 ng of standard **N** dissolved in 1.0 ml of a solution comprising 50.0 ng of internal standard. For **V,** the limit of detection was 0.924 ng/ml and the limit of determination was 3.080 ng/ml. That corresponds to 1.232 ng/ml of **V** in the serum sample. For **N**, the limit of detection was 0.030 ng/ml and the limit of determination was 1.001 ng/ml, which corresponds to 0.4 ng/ml of **N** in the serum sample.

Preliminary experiments with the use of UV detection provided unsatisfactory chromatograms with numerous peaks resulting from the biological background. The use of the fluorescence detector

Table 1
Precision and accuracy of the HPLC method of verapamil determination

Concentration of verapamil hydrochloride (ng/ml)	Inter-assay reproducibility		Intra-assay reproducibility			
	Measured *F*-factor (mean ± SD) [a]	CV (%)	Measured *F*-factor (mean ± SD) [a]	CV (%)	Measured concentration (ng/ml) [b]	Accuracy (%)
5	0.6750 ± 0.0305	4.52	0.6520 ± 0.0327	5.02	5.28	105.60
25	1.1730 ± 0.0416	3.55	1.1440 ± 0.0541	4.73	24.60	98.40
50	1.1779 ± 0.0645	3.62	1.7120 ± 0.0784	4.58	48.90	97.80
125	6.0100 ± 0.2981	4.96	5.8500 ± 0.2995	5.12	123.80	99.04
250	12.2100 ± 0.5461	4.47	11.8700 ± 0.6018	5.07	247.70	99.08

[a] *F*-factor: the ratio of peak area of verapamil hydrochloride to peak area of propranolol hydrochloride.
[b] Calculated from linear regression equation.

Table 2
Precision and accuracy of the HPLC method of norverapamil determination

Concentration of norverapamil (ng/ml)	Inter-assay reproducibility		Intra-assay reproducibility			
	Measured *F*-factor (mean ± SD) [a]	CV (%)	Measured *F*-factor (mean ± SD) [a]	CV (%)	Measured concentration (ng/ml)[b]	Accuracy (%) [b]
5	0.2100 ± 0.0152	7.23	0.2180 ± 0.0211	9.68	5.32	106.40
25	0.9900 ± 0.0360	3.64	0.9870 ± 0.0450	4.56	24.50	98.00
50	2.2670 ± 0.1133	4.99	2.2510 ± 0.1332	5.92	48.50	97.00
125	5.5277 ± 0.2650	4.79	5.3280 ± 0.2728	5.12	122.80	98.24
250	10.3430 ± 0.2637	4.39	10.2850 ± 0.5123	5.03	246.50	98.60

[a] *F*-factor: the ratio of peak area of norverapamil to peak area of propranolol hydrochloride.
[b] Calculated from linear regression equation.

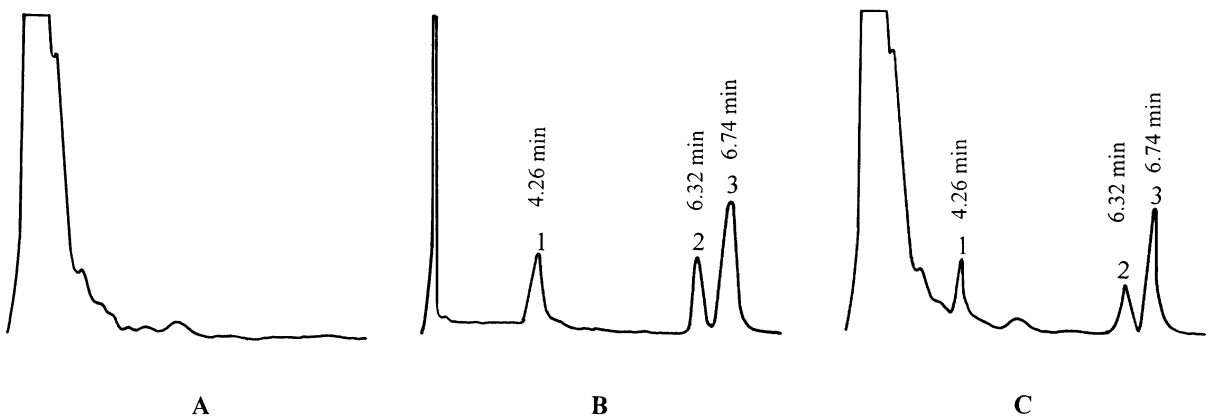

Fig. 1. Chromatograms of plasma samples; 1—propranolol hydrochloride (4.26 min), 2—norverapamil (6.32 min); 3—verapamil (6.74 min): (A) sample without drugs. (B) sample without drugs fortified with standard solutions, comprising 10.0 ng/ml of propranolol hydrochloride, 15.0 ng/ml of verapamil and 20.0 ng/ml of norverapamil. (C) sample of serum of a volunteer given a 40 mg *Staveran* tablet; concentration of verapamil is 38 ng/ml.

allowed us to achieve appropriate separation of analytes under the analytical conditions applied without any biological background interference. Fig. 1 presents an example chromatogram obtained for standard plasma without a drug (Fig. 1A) and with the analytes (Fig. 1B). The following retention times were noted: propranolol hydrochloride—4.26 min, **N**—6.32 min and **V**—6.74 min.

Recovery from plasma is very high at 92.12 and 89.58% for **V** and **N**, respectively.

Validation parameters are shown in Tables 1 and 2. From these results one can conclude that the precision of the proposed method is very high particularly taking into account the lack of autosampler. The method is fast and one chromatogram separation lasts about 8 min. Thirty to forty manual analyses were done per day. CV, for **V** did not exceed 5.12% and for **N** ranges from 3.64 to 9.68%.

The method was used to determine the concentration of **V** and **N** in plasma of healthy volunteers after buccal application of a home made drug formulation containing 20.0 mg of **V** as well as after oral administration of capsules comprising flotation pellets with 40.0 mg of **V**. These new drug formulations were designed to provide a more effective dozing of verapamil in comparison to the reference conventional tablets *Staveran* (Polpharma S.A., Starogard Gdański, Poland). In sum, for three analysed drugs, administered to volunteers by means of a crossover test, five hundred samples were analysed chromatographically. The chromatogram presented in Fig. 1C proves the suitability of the proposed method for pharmacokinetic and bioavailability studies of verapamil.

4. Conclusions

Parameters of validation prove that the precision of the method presented is very good. The method is fast, convenient and enables the performance (without an autosampler) of 30–40 analyses per day. It was successfully used in pharmacokinetic and bioavailability studies of **V** administration in alternative drug formulations to tablets: buccal and flotation ones.

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