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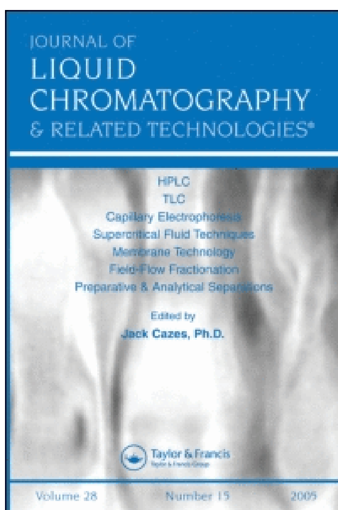

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SIMULTANEOUS HPLC ANALYSIS OF S- AND R-VERAPAMIL AND METABOLITES, S- AND R-NORVERAPAMIL IN HUMAN PLASMA

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ABSTRACT

This study aimed to develop a simple, sensitive, and accurate HPLC method for the simultaneous determination of verapamil and norverapamil enantiomers in human plasma. An internal standard (diphenhydramine) was used and separations of verapamil and norverapamil enantiomers were done at room temperature. Chromatographic separation was achieved using a Chiralcel® OD-RH column (5 µm, 4.6 mm i.d. x 15 cm, Daicel Chemical Industries Ltd., Tokyo, Japan) and a mobile phase consisting of 30 mM hexafluorophosphate and acetonitrile (66 : 34, v/v, pH 4.6). Detection of S- and R-verapamil and the metabolites, S- and R-norverapamil was accomplished with a fluorescence detector. The wavelengths of excitation and emission were set at 280 nm and 315 nm respectively.

The inter-day coefficients of variation (CV) of S-verapamil, R-verapamil, S-norverapamil, and R-norverapamil from extracted human plasma samples (at a high concentration, 250 ng/mL) were 9.8%, 5.3%, 4.3%, and 5.8%, respectively, while at a low concentration of 10 ng/mL, the CV were 15.9%, 17.5%, 15.6%, and 14.9%, respectively. The recovery was 117%, 117%, 110%, and 96.3% for S-verapamil, R-verapamil, S-norverapamil, and R-norverapamil respectively at low concentration (10 ng/mL) and

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was 95%, 108%, 124%, and 107%, at high concentration (250 ng/mL), respectively. The plasma samples were stable for at least three months after the samples were collected and stored at –20°C. The lower detection limit for each of S- and R-verapamil and the metabolites, S- and R-norverapamil in human plasma was 10 ng/mL. The present method is simple because it does not require a column-switching system or organic solvent as a component in the mobile phase, and the sample preparation is more economic as solid-phase extraction is not required. Thus, the assay described in this report is suitable for studying the pharmacokinetics and metabolism of verapamil.

INTRODUCTION

Verapamil, a calcium channel blocker, is widely used as a first line drug in the clinical treatment of angina pectoris, hypertension. and supraventricular arrhythmia.[1,2] Verapamil undergoes extensive biotransformation in human liver by *N*-demethylation and *O*-demethylation as well as *N*-dealkylation.[2-3] Both *in vivo* and *in vitro* evidence indicates *N*-demethylation with formation of norverapamil to be one of the major metabolic pathways.[4-5]

The metabolite norverapamil has been shown to have pharmacological activity similar to verapamil.[2] It produced about 20% of the coronary vasodilator activity of the parent drug when administered intra-arterially to dogs.[6] Both verapamil and norverapamil are chiral, and their pharmacokinetics is enantio selective.[5] The verapamil enantiomers also show different pharmacodynamic and pharmacokinetic properties with the S-verapamil being more pharmacologically active.[7-8] S-verapamil was reported to be 18 times more potent than R-verapamil in producing 10% PR-interval prolongation in humans.[8] The plasma clearance and apparent volume of disposition of S-verapamil are much higher than those of R-verapamil after intravenous administration.[7] The pharmacokinetic difference between the two enantiomers was even greater when determined after oral administration. The apparent oral clearance of S-verapamil was almost 5 times that of the R-verapamil and the bioavailability of R-verapamil was more than double that of S-verapamil.[9]

HPLC is a common technique used to determine verapamil and its metabolite norverapamil in biological samples.[10-12] As a number of drugs are marketed as racemates, more attention has therefore been directed toward the development and application of stereospecific chromatographic assays for *in vitro* and *in vivo* studies of racemic agents.[13-15] Verapamil is manufactured and administered as the racemate and most of the analytical techniques to date have not satisfactorily resolved and quantified the individual enantiomers for both the parent drug and its metabolites (Figure 1). Different chiral HPLC methods have

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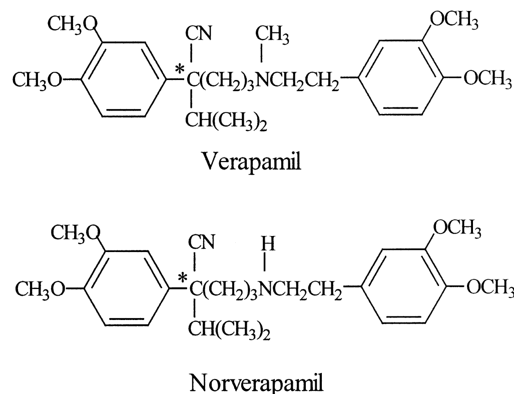

Figure 1. Chemical structures of verapamil and norverapamil with asymmetric carbon atom indicated by an asterisk.

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been developed for the quantitative analysis of verapamil and norverapamil enantiomers. These include the use of β-cyclodextrin, α_1-acid glycoprotein, ovomucoid, and a derivatized amylose column.[16-19] Some of these methods require elaborate systems consisting of several pumps and the connecting of achiral-chiral columns with a column-switching step,[9,18] or utilise a microwave-facilitated derivatization followed by resolution on an achiral reversed-phase column.[20]

EXPERIMENTAL

Materials and Chemicals

All chemicals and reagents were of analytical grade, including acetonitrile (HPLC-grade), orthophosphoric acid (85%), diethylether, and sodium hydroxide (BDH Chemicals, Poole, UK), diphenhydramine hydrochloride, and potassium hexafluorophosphate (Sigma Chemical Co., St. Louis, MO, USA), S-verapamil, R-verapamil, S-norverapamil, R-norverapamil (Knoll AG, Ludwigshafen, Germany). HPLC water used was double glass and MilliQ® filtered.

Standard Solutions of Verapamil and Norverapamil Enantiomers

Stock solutions of S- and R-verapamil and the metabolites, S- and R-norverapamil were initially prepared in water. From these stock solutions, known concentrations of S- and R-verapamil and the metabolites, S- and R-

norverapamil in drug-free human plasma were prepared. The standard curve for the assay covered the range of concentrations from 10 to 250 ng/mL. The internal standard, diphenhydramine (1 mg/mL), was prepared in water on each day of analysis.

Extraction of Verapamil and Norverapamil Enantiomers

To 1 mL of plasma sample was added 100 µL of NaOH (4 M) and 200 µL of internal standard (diphenhydramine, 1 mg/mL). The mixture was extracted with 7 mL of diethylether. The contents were shaken on a mechanical shaker for 20 min, and then centrifuged at 1500 g (2500 rpm, at 4°C) for 10 min. The ether layer was transferred into a tapered centrifuge tube containing 250 µL of 0.1% orthophosphoric acid and shaken for 20 min. After centrifugation at 1500 g (2500 rpm, at 4°C) for 10 min, the organic layer was aspirated and discarded. An aliquot (70 µL) of the acidic aqueous solution was injected onto the HPLC column for analysis.

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Chromatographic Conditions

R- and S-verapamil and the metabolites, R- and S-norverapamil were assayed by a reversed-phase HPLC method. A Chiralcel® OD-RH column (5 µm, 4.6 mm i.d. x 15 cm, Daicel Chemical Industries Ltd., Tokyo, Japan) was used. A mobile phase containing 30 mM hexafluorophosphate and acetonitrile (66 : 34, v/v, pH 4.6) was used. The flow rate was set at 1 mL/min. Separations were done at room temperature. Detection of S- and R-verapamil and the metabolites, S- and R-norverapamil was accomplished with a fluorescence detector (Hitachi, Tokyo, Japan). The wavelengths of excitation and emission were set at 280 nm and 315 nm, respectively. 70 µL of the acidic aqueous solution was injected onto the column by a Jasco AS-950 Intelligent autosampler (Jasco Corporation, Tokyo, Japan). Chromatograms of parent drug and metabolites were recorded by a Hitachi D-2500 integrator (Hitachi, Tokyo, Japan).

Recovery Study

Absolute recovery of S- and R-verapamil and S- and R-norverapamil was assessed at three different concentrations (10, 25, and 250 ng/mL) by comparing the peak heights from 4 or 5 different standard samples with those obtained by direct injection of the same amount of the pure compound solution prepared in water. The absolute recovery of the internal standard was assessed using the same procedure.

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Stability Study

Stability of S- and R-verapamil and S- and R-norverapamil in plasma samples stored frozen at -20°C up to 3 months was evaluated. This was carried out by determining plasma concentrations of the parent drug and its metabolites in the fresh plasma samples and in the same samples after storage at −20°C for 3 months.

RESULTS

Separation and Resolution of Verapamil and Norverapamil Enantiomers

A chromatogram of human blank plasma (i.e. drug-free plasma) extract is shown in Figure 2A. None of the endogenous peaks detected interfere with the assay. Figure 2B illustrates a representative chromatogram of an extracted plas-

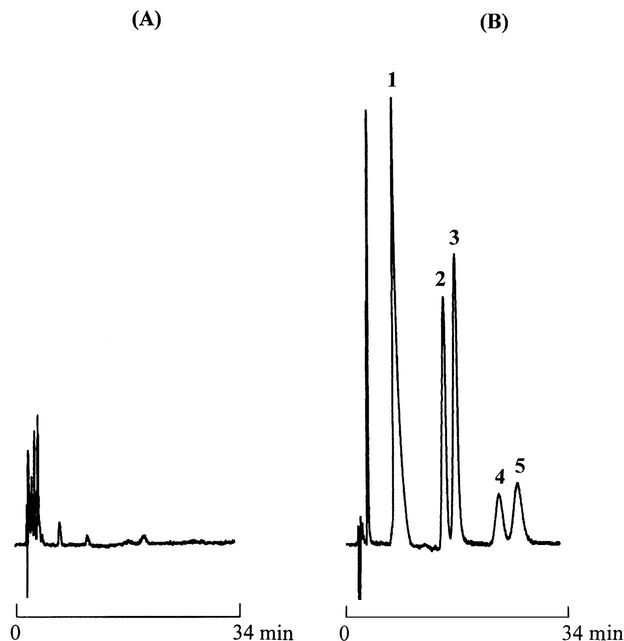

Figure 2. Chromatograms of an extracted blank plasma (A), and (B) an extracted plasma sample obtained from a healthy volunteer 8 h after an oral dose of 120 mg verapamil (Isoptin® SR, Knoll Australia Pty Ltd). Peak identification: 1 = internal standard, diphenhydramine (1 mg/mL); 2 = R-norverapamil (59.9 ng/mL); 3 = R-verapamil (71.5 ng/mL); 4 = S-verapamil (22.5 ng/mL); and 5 = S-norverapamil (23.5 ng/mL).

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ma sample obtained from a volunteer 8 h after an oral administration of 120 mg verapamil. Under the chromatographic conditions used, the peaks for internal standard (diphenhydramine), R-norverapamil, R-verapamil, S-verapamil, and S-norverapamil were well resolved and had retention times of 8.3, 17.1, 19.3, 26.4, and 29.4 min, respectively (Figure 2B). The lower detection limits for S- and R-verapamil and the metabolites, S- and R-norverapamil in human plasma were 10 ng/mL based on a signal to noise ratio of 3:1.

Calibration Curves of Verapamil and Norverapamil Enantiomers

Under the conditions described (see Materials and Methods) the calibration curves (Figure 3) of S- and R-verapamil and the metabolites, S- and R-norverapamil were linear in the concentration range, 10 to 250 ng/mL. The correlation coefficients were good with r^2 values of 0.9983, 0.9998, 0.9998, and 0.9882 for R-norverapamil, R-verapamil, S-verapamil, and S-norverapamil, respectively.

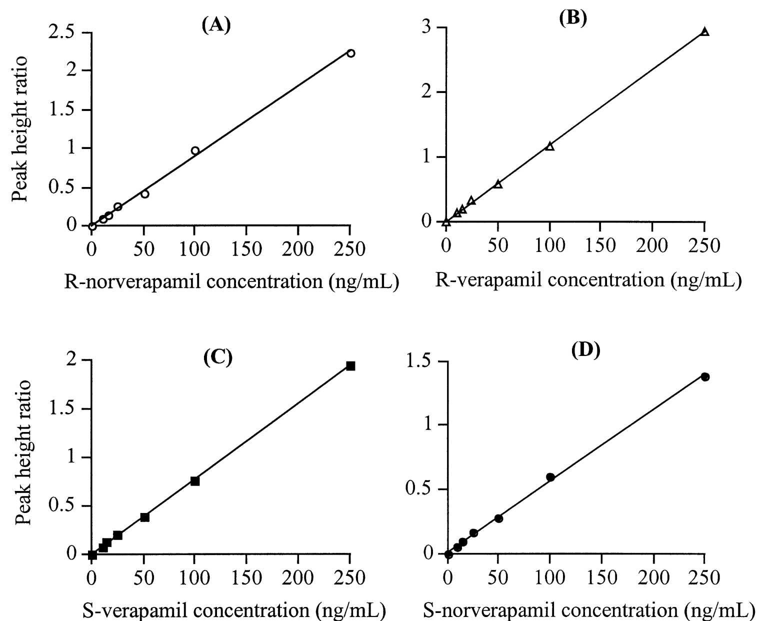

Figure 3. Calibration curves of (A) R-norverapamil, (B) R-verapamil, (C) S-verapamil, and (D) S-norverapamil by a plot of the peak height ratio (drug or metabolite/internal standard ratio) *vs* plasma drug or metabolite concentration.

Table 1

Recovery of Individual Enantiomers of Verapamil and Norverapamil from Human Plasma at Three Different Concentrations*

Concentration (ng/mL)	Recovery % S-Verap.	R-Verap.	S-Norverap.	R-Norverap.
10	117 ± 7	117 ± 7	110 ± 13	96.3 ± 7
25	104 ± 16	107 ± 17	111 ± 10	90.6 ± 2
250	95 ± 6	108 ± 3	124 ± 8	107 ± 4

* (n = 4).

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Recovery of Verapamil and Norverapamil Enantiomers

The absolute recovery of R-norverapamil, R-verapamil, S-verapamil, as well as S-norverapamil, from plasma at three different concentrations, is listed in Table 1. The recovery was 96.3%, 117%, 117%, and 110% for R-norverapamil, R-verapamil, S-verapamil, and S-norverapamil respectively at low concentration (10 ng/mL). At high concentration (250 ng/mL) the recovery was 107%, 108%, 95%, and 124% for R-norverapamil, R-verapamil, S-verapamil, and S-norverapamil, respectively.

Reproducibility and Accuracy of Verapamil and Norverapamil Assays

The within-day (intra-day) reproducibility of the assay was evaluated by analysing four plasma samples at different concentrations, i.e. 10, 25, 50, and 250 ng/mL. The results of this evaluation are listed in Table 2. Good reproducibility was found with coefficients of variation (CV) 3.0, 6.4, 11.0, and 3.5% for S-verapamil, R-verapamil, S-norverapamil, and R-norverapamil, respectively, at a low concentration (10 ng/mL). At a high concentration of 250 ng/mL, the CV values were 3.1, 2.0, 2.1, and 4.0% for S-verapamil, R-verapamil, S-norverapamil, and R-norverapamil, respectively. The accuracy of the assay was in the range 106 - 120% (Table 2).

Table 3 showed the inter-day variation for the HPLC assay of S-verapamil, R-verapamil, S-norverapamil, and R-norverapamil from extracted human plasma samples. At a high concentration (250 ng/mL) the CV were 9.8%, 5.3%, 4.3%, and 5.8% for S-verapamil, R-verapamil, S-norverapamil, and R-norverapamil, respectively. At a low concentration of 10 ng/mL, the coefficients of variation became slightly higher than those seen at the high concentration with values ranging from 14.9% for R-norverapamil to 17.5% for R-verapamil

Table 2

Reproducibility and Intra-Day Accuracy of the Simultaneous HPLC Assay for S- and R- Enantiomers of Verapamil and Norverapamil in Human Plasma

Concentration Used (ng/mL)	Observed[a] Concentration (ng/mL)	CV[b] (%)	Accuracy[c] (%)
S-Verapamil			
10	11.3 ± 0.3	3.0	113
25	29 ± 3.9	13.0	116
50	53 ± 0.6	1.1	106
250	279 ± 8.6	3.1	111
R-Verapamil			
10	11.8 ± 0.7	6.4	118
25	30 ± 3.9	13.0	120
50	56 ± 1.7	3.0	112
250	278 ± 5.6	2.0	111
S-Norverapamil			
10	11.6 ± 1.3	11.0	116
25	33 ± 1.6	4.9	132
50	56 ± 2.9	5.0	113
250	281 ± 7.5	2.5	112
R-Norverapamil			
10	10.9 ± 0.4	3.5	109
25	29 ± 0.6	2.2	116
50	55 ± 1.9	3.4	110
250	281 ± 11	4.0	112

[a] Results given are mean ± SD (n=4). [b] CV is a coefficient of variation.

[c] $\text{Accuracy } (\%) = \frac{\text{observed concentration}}{\text{concentration used}} \times 100$

(Table 3). Overall, accuracy of the assay was found to be between 94 to 120% at the concentrations of analytes of interest (Table 3).

Stability of Verapamil and Norverapamil Enantiomers in Plasma Samples

The stability of S- and R-verapamil as well as norverapamil in seven plasma samples was studied on two separate occasions, i.e., fresh samples and up to 3

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Table 3

Reproducibility and Inter-Day Accuracy of the HPLC Assay for Enantiomers of Verapamil and Norverapamil in Human Plasma

	Observed Concentration (ng/mL)[a]							
Conc. ng/mL	**S-Verap.**	**CV %**	**R-Verap.**	**CV %**	**S-Norverap.**	**CV %**	**R-Norverap.**	**CV[c] %**
10	9.2 ± 1.5 (92%)[b]	15.9	9.3 ± 1.6 (93%)	17.5	9.4 ± 1.5 (94%)	15.6	10.6 ± 1.6 (106%)	14.9
25	29 ± 2.4 (118%)	8.1	26 ± 2.9 (105%)	11.3	30 ± 0.6 (120%)	2.0	30 ± 1.3 (120%)	4.2
50	54.9 ± 4.6 (110%)	8.4	51 ± 1.7 (102%)	3.3	57.9 ± 6.3 (116%)	10.9	59 ± 4.8 (118%)	8.2
100	112 ± 10 (112%)	9.0	99.6 ± 9.5 (100%)	8.5	116 ± 7 (116%)	6.0	114 ± 4.9 (114%)	4.3
250	262 ± 26 (105%)	9.8	236 ± 12 (94%)	5.3	238 ± 10 (95%)	4.3	248 ± 14.5 (99%)	5.8

[a] Values are given as mean ± SD (n = 4). [b] Values in the parenthesis are accuracy determined as: Accuracy (%) $= \frac{\text{observed concentration}}{\text{concentration used}} \times 100$. [c] CV is a coefficient of variation.

months storage at –20°C. Table 4 shows the results of the analysis of fresh and frozen (stored at –20°C for 3 months) plasma samples. When the samples were collected after a single dose of verapamil, the mean plasma concentrations of S-, R-verapamil, as well, as S-, R-norverapamil in the plasma samples were 29 ± 19 ng/mL, 97 ± 56 ng/mL, 32 ± 9 ng/mL, and 73 ± 25 ng/mL, respectively. After the same samples were stored at -20°C for 3 months, the mean plasma concentrations of S-, R-verapamil, as well as, S-, R-norverapamil were 24 ± 18 ng/mL, 85 ± 56 ng/mL, 32 ± 8 ng/mL, and 67 ± 26 ng/mL, respectively. There were no significant differences between the plasma concentrations of each of the individual enantiomers of both verapamil and norverapamil for fresh samples and the frozen stored samples ($P > 0.05$).

Table 4

Stability of Verapamil and Norverapamil Enantiomers in Human Plasma Samples

Sample Number	Concentration on Date of Assay (ng/mL) R-Norverap.	R-Verap.	S-Verap.	S. Norverap.
1	92[a] (76)[b]	97 (73)	26 (18)	31 (30)
2	116 (108)	204 (179)	65 (55)	44 (47)
3	67 (63)	68 (61)	18 (16)	24 (28)
4	81 (91)	140 (150)	42 (44)	46 (40)
5	47 (42)	46 (35)	12 (10)	22 (23)
6	56 (49)	69 (57)	21 (16)	32 (31)
7	50 (41)	58 (42)	16 (12)	28 (27)

[a] Analysis of freshly collected sample. [b] Analysis of sample after storage at -20°C for 3 months.

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DISCUSSION

The evaluation of verapamil pharmacokinetics with regard to metabolism and disposition after oral administration requires a sufficiently sensitive and selective analytical method for the determination not only of verapamil, but also of the major metabolites in the plasma of patients undergoing oral therapy. The contribution of active metabolites of antiarrhythmic agents to unanticipated drug efficacy or toxicity, as well as their accumulation with abnormalities of renal or hepatic function, has been reviewed.[21] Impaired hepatic function has been demonstrated to prolong the elimination phase after intravenous verapamil administration. The reduction of systemic clearance was 3-fold in subjects with liver dysfunction.[22] In patients with normal hepatic and renal function, the elimination of both verapamil and norverapamil has been found to be prolonged during chronic oral therapy.[23]

Verapamil is used as a racemic mixture of R- and S-verapamil. The S-verapamil has been shown to be preferentially metabolised especially after oral dosing, thereby leading to the predominance of R-verapamil in plasma.[8] Therapeutic plasma concentration range of verapamil is 100-400 ng/mL.[2] A discrepancy exists between the duration of the electrophysiological and hemodynamic effects of verapamil and its concentration in plasma. The possibility that this discrepancy may be related to the concentration of metabolites has been proposed.[24] Measurements of plasma concentrations of verapamil and metabolite enantiomers during oral therapy, therefore, may help decrease the frequency of adverse drug effects and improve the likelihood of successful therapy.

Under the chromatographic conditions described in the present study, the enantiomers of R-, and S-verapamil and the metabolites, R- and S-norverapamil were separated with good resolution. The standard curves for verapamil and norverapamil enantiomers (Figure 3) were linear with a very good correlation coefficient (r^2).

The reproducibility of the HPLC method developed was good, as indicated by low intra- and inter-day variation with an acceptable accuracy. The coefficients of variation for the assay at the low concentration (10 ng/mL) were slightly higher than a normal acceptable CV (CV ≤ 10%), but they are still below a 20% acceptable CV for the minimum quantifiable concentration (MQC). The method is also proven to be a good assay with high extraction recovery for both the parent drug and its metabolite (norverapamil). Stability of the plasma samples after storage (at –20°C, for 3 months) was established which provides a feasibility of analysing the samples (such as those from pharmacokinetic studies) within 3 months of sample collection.

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The present assay also has advantages over previously reported HPLC methods as it employed a back-extraction technique, so sample preparations (e.g. solid-phase extraction, evaporation) are not required. Thus, the assay can be achieved in a relatively short time and at lower cost. The enantiomeric separation of the verapamil and norverapamil enantiomers is accomplished at room temperature. A good baseline resolution of the enantiomers is achieved by using a simple aqueous mobile phase instead of using organic solvent or corrosive compound in the mobile phase. Under the conditions used the column life may be longer and suitable for a large number of sample analyses for clinical studies. The present technique allows the simultaneous determination of the above enantiomers without using diasteriomeric derivatives and therefore can avoid errors caused by different reaction rates of the enantiomers with the chiral reagent, or by racemization during derivatization.[24]

In summary, the HPLC method described is a reliable and simple technique, providing sufficient sensitivity and precision for determination of both verapamil and norverapamil enantiomers. Thus it is suitable to be used for both therapeutic monitoring (therapeutic range of verapamil plasma concentrations between 100 and 400 μg/L)[2] and for pharmacokinetic studies.

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