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Stereoselective determination of verapamil and norverapamil by capillary electrophoresis

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Abstract

An analytical method has been developed to determine simultaneously the verapamil and norverapamil enantiomers in human plasma using capillary electrophoresis. Among the cyclodextrins tested as chiral selector, only trimethyl-β-cyclodextrin was suitable to resolve the four enantiomers. The analysis was achieved in less than 10 min. Selectivity, linearity, precision and accuracy were evaluated before the chiral method was successfully implemented for routine use to simultaneously determine the four enantiomers in several thousands of human plasma samples. The robustness of the capillary electrophoretic method and its suitability were demonstrated by the coefficients of variation which were lower than 11%, even at the limit of quantification (2.5 ng/ml), for the analysis of more than one hundred quality control samples.

1. Introduction

Chiral assay analysis by capillary zone electrophoresis with cyclodextrin in the electrolyte was first described by S. Fanali [1]. In the presence of dimethyl-β-cyclodextrin, the separation of epinephrine, norepinephrine and isoproterenol enantiomers was performed in less than 6 min. The use of the appropriate cyclodextrin in the electrolyte allows the formation of a complex between the enantiomers and the cyclodextrin. The resolution of the enantiomers depends on the differential affinities of the enantiomers for the chiral selector [2]. Numerous parameters such as cyclodextrin type, cyclodextrin concentration, pH of the electrolyte, capillary length and buffer concentration modify the resolution of the enantiomers. For instance, the influence of cyclodextrin type was demonstrated for the enantiomers of terbutaline [3], barbiturates [4], propranolol [2,3,5] as well as atenolol and pilocarpine [5].

Verapamil, like many other synthetic drugs, is a racemic mixture. It has antiarrhythmic, antianginal and antihypertensive properties. Although verapamil is well absorbed by the gastrointestinal tract, it has a low oral bioavailability due to its large first pass metabolism in the liver. Verapamil has several metabolites, including norverapamil which has some therapeutic activity. The enantiomers of both verapamil and norverapamil have different pharmacokinetic

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and pharmacodynamic profiles [6] thus justifying the development and the validation of stereoselective analytical methods in order to adequately assess the bioequivalence of different verapamil formulations.

Reported methods for the simultaneous determination of the four enantiomers by high-performance liquid chromatography (HPLC) have been based on the preliminary separation of verapamil and norverapamil from each other and from plasma components, followed by the enantiomeric resolution on a chiral phase using a column-switching system [7,8] or columns connected in series [9]. Chiral separations by HPLC suffer from low separation efficiencies and lack of baseline resolution. They are also time-consuming. Capillary electrophoresis (CE) is a promising alternative, combining high efficiency and versatility. Stereoselective analysis of verapamil [10] and of both verapamil and norverapamil [11] by CE have been described. Because the current verapamil and norverapamil chiral assay was developed to quantitate the four enantiomers in human plasma samples obtained from pharmacokinetic studies, complete resolution, automation, sensitivity, specificity against endogenous compounds, precision, accuracy and robustness were required. Here we report the development and validation of an analytical procedure for the simultaneous determination of *R*-verapamil, *S*-verapamil, *R*-norverapamil and *S*-norverapamil in human plasma.

2. Experimental

2.1. Instrumentation

The automated analyses were conducted using a Phoresis 1000 instrument (Spectra-Physics, San Jose, CA, USA). The internal diameter of the fused-silica capillaries was 75 μm. Initial studies used a capillary length to the detector of 36 cm and the length to the detector was reduced to 18 cm in later studies. Detection with a time constant of 4 s was by on-capillary UV absorbance at 200 nm. The automated CE instrument was controlled by Phoresis software running on an IBM PS/2 Model 70386 computer. Collected data were integrated with Ingrad System software (G.D. Searle, Skokie, IL, USA). A run potential of 12 kV was used resulting in a current of 90–100 μA through the capillary. The capillary temperature was maintained at 15°C by a Pelletier element.

2.2. Chemicals

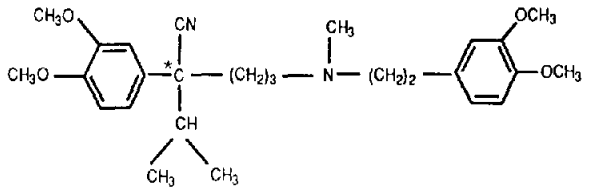
All chemicals were of analytical grade. Isopropanol, methanol, ethanol, hexane, phosphoric acid (85%), and sodium hydroxyde were from Merck (Darmstadt, Germany). Trimethyl-β-cyclodextrin, dimethyl-β-cyclodextrin and γ-cyclodextrin were purchased from Sigma Chemical Co. (Saint-Louis, MO, USA). Hydroxypropyl-β-cyclodextrin was from Janssen Chemica (Beerse, Belgium). Verapamil hydrochloride and norverapamil hydrochloride (Fig. 1) were provided by G.D. Searle (Skokie, IL, USA). Gallopamil hydrochloride (Fig. 1), used as internal standard, was obtained from Recordati (Milano, Italy).

2.3. Electrophoresis procedures

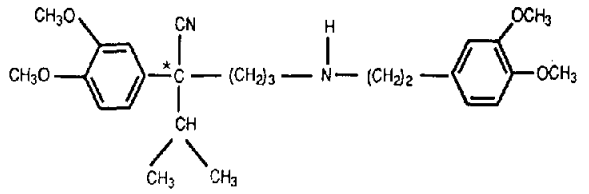
The capillary of the Phoresis 1000 was successively washed for 1 min each with 0.1 *M* sodium hydroxyde and with a phosphate solution (4 ml phosphoric acid in 1 l water) brought to pH 2.5 with 1 *M* NaOH. The capillary was then filled with the phosphate solution containing 60 m*M* trimethyl-β-cyclodextrin for 2 min. The sample was injected by electromigration at 10 kV for 7 s. The washes and buffer replacement were repeated before each analysis. The run potential was 12 kV and absorbance was monitored at 200 nm.

2.4. Sample preparation

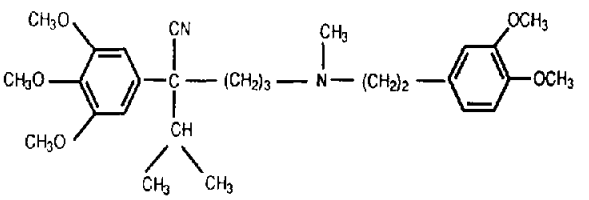
To 1.0 ml human plasma in a screw-capped glass centrifuge tube were added 0.1 ml of gallopamil solution (1 mg/ml), 1 ml of 0.1 *M* sodium hydroxyde and 5 ml of an hexane–isopropanol mixture (90/10, v/v). After vortex-mixing for 30 s and centrifugation for 10 min at



VERAPAMIL



NORVERAPAMIL



GALLOPAMIL

Fig. 1. Chemical structures of verapamil, its active metabolite norverapamil and the internal standard, gallopamil. * = position of the asymmetric carbon.

1500 *g*, the organic phase was transferred into a clean glass tube and evaporated to dryness under a stream of nitrogen. The residue was reconstituted in 0.1 ml methanol–water (25/75, v/v). The sample was transferred to a suitable insert and centrifuged before CE analysis.

3. Results

3.1. Development of the chiral assay

The verapamil and norverapamil enantiomers could be readily resolved using trimethyl-β-cyclodextrin as chiral additive while the separation was incomplete in the presence of γ-cyclodextrin, dimethyl-β-cyclodextrin and hydroxypropyl-β-cyclodextrin. With an uncoated (fused-silica) capillary 36 cm long and the electrolyte at pH 2.5 with 60 m*M* trimethyl-β-cyclodextrin, the migration times were *ca.* 20 min (Fig. 2A). After a slight modification of the capillary cassette, short ($l = 18$cm) capillaries could be used and resolution of the four enantiomers was obtained in approximately 10 min. (Fig. 2B).

Under acidic conditions, verapamil and norverapamil are positively charged and migrate towards the cathode. The pH range 2.5–5.5 was investigated for the optimal acidity of the electrolyte. Migration times and the resolutions were similar at pH 2.5 and 3.5. At higher pH migration times were reduced but resolution between the four enantiomers also decreased (Fig. 3). An increased electroosmotic flow due to higher pH reduced the migration times and, as a consequence, the time needed to achieve resolution.

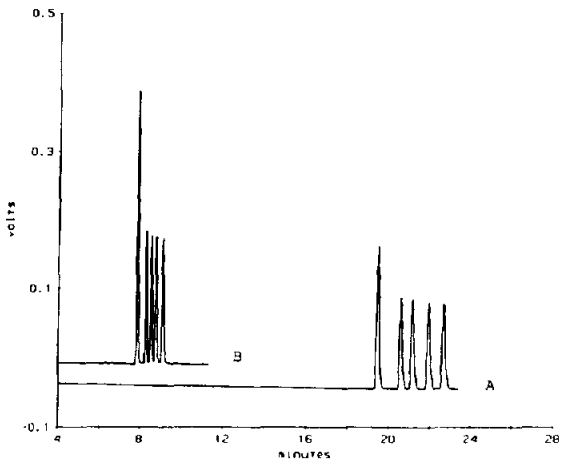


Fig. 2. Influence of the capillary length on the migration times of the verapamil and norverapamil enantiomers. Eluted peaks: internal standard, *R*-verapamil, *R*-norverapamil, *S*-verapamil and *S*-norverapamil, respectively. Instrument: Phoresis 1000; Electrolyte: 60 m*M* phosphate buffer pH 2.5 with 60 m*M* trimethyl-β-cyclodextrin; Capillary: 75-μm fused-silica; Injection: electromigration at 10 kV for 5 s; Electrophoresis: constant voltage 12 kV; Detection: UV absorbance at 200 nm. A: capillary length to detector = 18 cm. B: capillary length to detector = 36 cm.

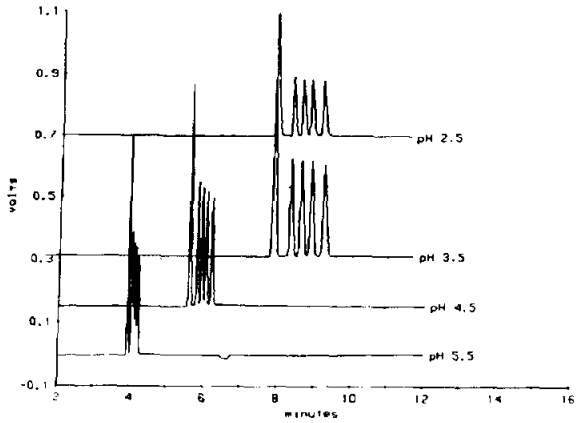


Fig. 3. Influence of the pH (from 2.5 to 5.5) on the migration times of the verapamil and norverapamil enantiomers. Eluted peaks: internal standard, *R*-verapamil, *R*-norverapamil, *S*-verapamil and *S*-norverapamil, respectively. Instrument: Phoresis 1000; Electrolyte: 60 m*M* phosphate buffer with 60 m*M* trimethyl-β-cyclodextrin; Capillary: 18 cm × 75 μm I.D. fused-silica; Injection: electromigration at 10 kV for 5 s; Electrophoresis: constant voltage 12 kV; Detection: UV absorbance at 200 nm.

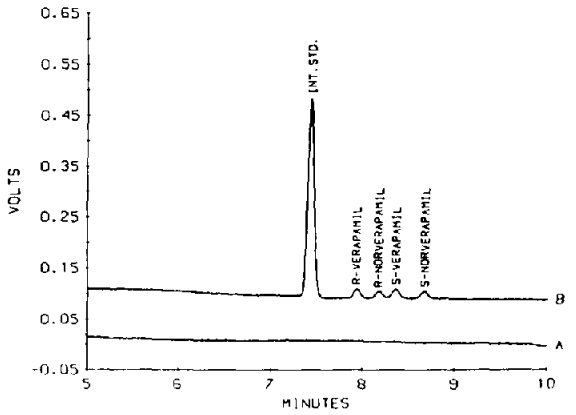


Fig. 4. Typical electropherograms of verapamil and norverapamil chiral assay for: A = drug-free human plasma (1 ml). B = drug-free human plasma (1 ml) spiked with 2.5 ng of each enantiomer and 100 ng of internal standard. Electrolyte: 60 m*M* phosphate buffer pH 2.5 with 60 m*M* trimethyl-β-cyclodextrin; Capillary: 18 cm × 75 μm I.D. fused-silica; Injection: electromigration at 10 kV for 7 s; Electrophoresis: constant voltage 12 kV; Detection: UV absorbance at 200 nm.

This conclusion is supported by restored resolution when the capillary length was increased from 18 cm to 36 cm.

In order to reach adequate sensitivity and selectivity drugs were loaded in the capillary by electromigration instead of hydrodynamic injection. Applying a high voltage for a few seconds greatly enhances the amount of drug introduced into the capillary. However, this injection mode is less reproducible, especially if the ionic strength of the injected solution is not constant. The variability associated with this injection method was compensated by choosing an internal standard, gallopamil, with electrophoretic properties very close to that of the monitored drugs.

The specificity against blank plasma was demonstrated by the absence of interfering compounds at the migration times of the monitored drugs. As a concentration of 2.5 ng of each enantiomer per ml human plasma was distinctly detected (Fig. 4), the sensitivity of the assay was judged sufficient for the purpose of pharmacokinetic studies.

3.2. Validation of the chiral assay

Assay validation seeks to define the selectivity, sensitivity, linearity, precision and accuracy of the bioanalytical method [12].

Selectivity against endogenous compounds was confirmed using six independent samples of human plasma. No interference was observed at the migration times of the internal standard and the monitored drugs.

Known amounts of verapamil hydrochloride and norverapamil hydrochloride (2.5–250 ng of each enantiomer) and internal standard (100 ng) were added to drug-free plasma to establish calibration curves for peak-area ratios. For each calibrator a response factor (calibrator concentration/peak-area ratio) was calculated and, for each calibration, the mean response factor was used to determine the amount of drug in each sample. The coefficients of variation on the mean response factor, typically between 3% and 6%, indicated the good relation between peak-area ratio and drug concentration.

Precision and accuracy were evaluated from quality control samples prepared by adding ver-

apamil hydrochloride and norverapamil hydrochloride to drug-free human plasma. A recent consensus document on acceptance criteria for precision and accuracy in bioanalytical methods [13] states: "not more than 15% coefficient of variation for precision and not more than 15% deviation from the nominal value for accuracy. However, at the limit of quantification, 20% is acceptable for both precision and accuracy . . .". The limit of quantification of 2.5 ng/ml for each of the four enantiomers is supported by coefficients of variation lower than 15% and analytical recoveries between 91% and 100% (Table 1). At higher concentrations, the coefficients of variation and the analytical recoveries were also in agreement with the acceptance criteria for within-day and between-day precision and accuracy (Table 1).

The assay performance was confirmed by the quality control data obtained during the analysis of a large series of plasma samples from a clinical study. A typical electropherogram for a patient who received a single 180-mg dose of sustained release verapamil is shown in Fig. 5. The coefficients of variation of more than one hundred QC samples at each of the four QC concentrations for each enantiomer were between 6.1% and 10.6%, well within the acceptance criteria; the analytical recoveries were between 92.5% and 99.9%, demonstrating the assay accuracy (Fig. 6). These results include inter-analyst and between instrument variability.

4. Discussion and conclusions

Analysis of drugs at low concentrations in complex matrices like biological fluids is one of

Table 1
Before-study validation: within-day and between-day precision and accuracy of the verapamil and norverapamil chiral CE assay

Theoretical concentration (ng/ml)	Within-day				Between-day			
	Mean ± S.D. (ng/ml)	*n*	C.V. (%)	A.R. (%)	Mean ± S.D. (ng/ml)	*n*	C.V. (%)	A.R. (%)
R-Verapamil hydrochloride								
2.5	2.36 ± 0.21	5	8.9	94.4	2.47 ± 0.22	7	9.1	98.8
10	9.05 ± 0.41	6	4.5	90.5	9.70 ± 0.44	7	4.6	97.0
100	92.3 ± 2.02	6	2.2	92.3	97.0 ± 7.21	7	7.4	97.0
250	235 ± 6.90	6	2.9	94.0	241 ± 15.0	7	6.2	96.4
S-Verapamil hydrochloride								
2.5	2.36 ± 0.26	5	11	94.4	2.46 ± 0.23	7	9.2	98.4
10	9.21 ± 0.53	6	5.7	92.1	9.48 ± 0.55	7	5.8	94.8
100	93.7 ± 2.15	6	2.3	93.7	98.3 ± 8.75	7	8.9	98.3
250	238 ± 6.80	6	2.8	95.2	247 ± 25.6	7	10.4	98.8
R-Norverapamil hydrochloride								
2.5	2.29 ± 0.25	5	11	91.6	2.48 ± 0.34	7	13.5	99.2
10	9.29 ± 0.39	6	4.2	92.9	10.1 ± 0.94	7	9.3	101
100	98.9 ± 4.06	6	4.1	98.9	105 ± 8.75	7	8.3	105
250	259 ± 21.5	6	8.3	103.6	254 ± 17.0	7	6.7	102
S-Norverapamil hydrochloride								
2.5	2.51 ± 0.30	5	12	100.4	2.39 ± 0.19	7	7.9	95.6
10	9.91 ± 0.42	6	4.2	99.1	9.74 ± 1.11	7	11.4	97.4
100	108 ± 5.54	6	5.1	108	110 ± 11.7	7	10.6	110
250	282 ± 21.3	6	7.6	113	265 ± 22.3	7	8.4	106

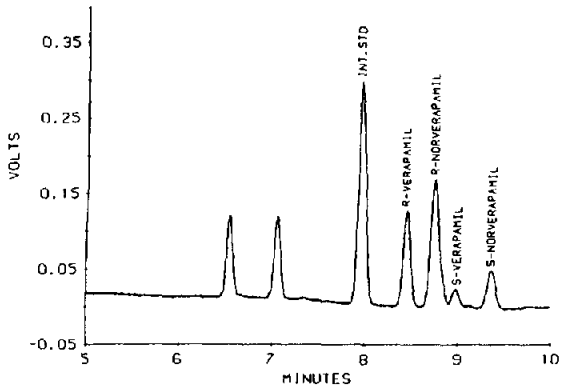


Fig. 5. Typical electropherogram of verapamil and norverapamil chiral determination in plasma (1 ml) from patient who received a single 180 mg dose of sustained release verapamil. Analytical conditions as in Fig. 4.

the major challenges of capillary electrophoresis. Micellar electrokinetic capillary chromatography without sample pretreatment has been used for some drugs [14–18]. The direct determination of cefpiramide [14] and of buthionine sulfoximine [17] had excellent precisions; coefficients of variation were less than 10%. However, with this approach the limit of quantification of *ca.* 10 to 20 μg/ml remains too high to study the pharmacokinetics of many drugs. Solid-phase and liquid–liquid extraction to clean-up samples before CE analysis has been used on biological samples for drug monitoring and quantification [10,19–24]. Although the number of reports describing chiral separation by CE is steadily increasing, stereoselective analyses biological fluids by CE

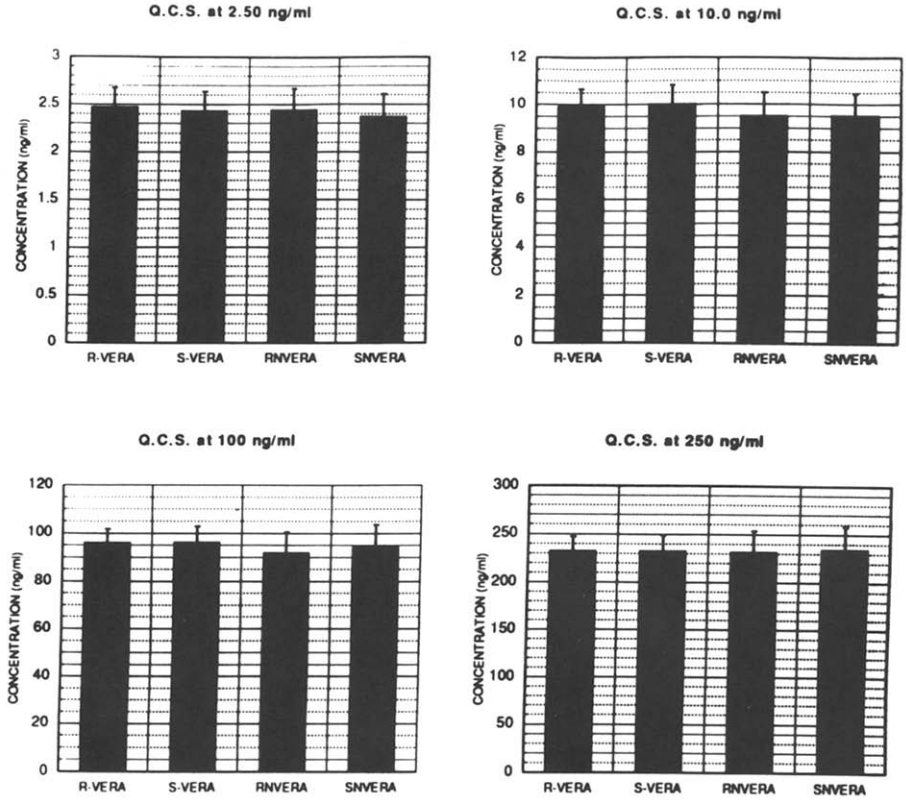


Fig. 6. Within-study validation: between-day precision and accuracy of the verapamil and norverapamil chiral CE assay. (Mean ± S.D., n = 124).

are still scarce. Recently chiral determinations for bupivacaine in serum [10] and cicletanine in plasma [24] were reported. In both assays, liquid–liquid extraction was used to clean-up the samples and as concentration step before the CE analysis. However the sensitivity of 10 ng/ml for the cicletanine enantiomers or of 2.5 ng/ml for the verapamil and norverapamil enantiomers was mainly achieved through the injection of solutions of low conductivity relative to the electrolyte leading to on-capillary peak concentration, also known as sample stacking [25].

Verapamil and norverapamil enantiomers have been quantified in our laboratory using CE in a large series of quality control samples over a two-month period. The excellent precision and accuracy obtained during this study by the six analysts running the assays confirmed the applicability of CE for stereoselective drug determination in biological fluids. Sensitivity, precision, accuracy and robustness were demonstrated in an assay showing a high power of separation.

This chiral CE validated method has been successfully used for routine analysis of several thousands of verapamil plasma analyses generated during pharmacokinetic studies.

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