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Validation of a method for the determination of (R)-warfarin and (S)-warfarin in human plasma using LC with UV detection

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Abstract

A sensitive and selective chiral high-performance liquid chromatography (HPLC) method was developed for the determination of (R)-warfarin and (S)-warfarin in human plasma. (R)- and (S)-warfarin and the internal standard (oxybenzone) were extracted from human plasma that had been made acidic with 1 N sulfuric acid into ethyl ether. The ethyl ether layer was removed and evaporated, and the residue was reconstituted in 200 μl of acetonitrile. A 50-μl aliquot was injected onto the HPLC system. Separation was achieved on a β-cyclodextrin column (250 × 4.6 mm, 5 μm) with a mobile phase composed of acetonitrile:glacial acetic acid:triethylamine (1000:3:2.5, v/v/v). Detection was by ultraviolet absorbance at 320 nm. Late-eluting peaks were diverted from the analytical column by using a β-cyclodextrin precolumn (50 × 4.6 mm, 5 μm) and a column switching device. The retention times of (R)- and (S)-warfarin and the internal standard were approximately 7.7, 6.9 and 4.0 min, respectively. The run time was 15 min. The assay was linear in concentration ranges of 12.5–2500 ng/ml for (R)- and (S)-warfarin in human plasma. The analysis of quality control samples for (R)- and (S)-warfarin (25.0, 400 and 2000 ng/ml) demonstrated excellent precision with relative standard deviations (R.S.D.) for (R)-warfarin of 10.9, 2.8, and 2.8%, respectively ($n = 18$), and for (S)-warfarin of 7.0, 2.4 and 2.6%, respectively ($n = 18$). The method was accurate with all overall ($n = 18$) mean concentrations being less than 6.0% from nominal at all quality control sample concentrations.

Keywords: (R)-Warfarin; (S)-Warfarin; Oxybenzone; Human plasma; Chiral separation; HPLC; β-cyclodextrin

1. Introduction

Thrombosis, the formation of blood clots in the circulation, can occur in both the arteries and veins. The causes can be of multiple origins, including an induction of platelet aggregation, an increase in platelet adhesiveness, or an increase in the activation of clotting factors, such as prothrombin. Thrombosis can severely reduce or stop blood flow to or from critical organs, resulting in permanent damage or death [1].

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Warfarin is an anticoagulant prescribed to treat this condition. It is an antagonist of vitamin K, and inhibits the synthesis of prothrombin by inhibiting vitamin K epoxide reductase and vitamin K reductase, which leads to a decrease in the ability of the blood to form clots [2,3]. Warfarin is a racemic compound, with (R)- and (S)-forms (Fig. 1). (S)-Warfarin is reported to have two to five times the anticoagulant potency of (R)-warfarin and is more quickly metabolized than (R)-warfarin [4]. A quick, rugged method was needed to determine the levels of (R)- and (S)-warfarin in human plasma.

Several methods for the quantitation of (R)- and (S)-warfarin in human plasma exist. A recent work utilizes microbore LC with peak compression to enhance enantiomer separation [5]. Analysis times were reduced to approximately 18 min (with very good resolution). The work presented here is based on a more conventional HPLC separation [6]. Enantiomeric separation took place on a β-cyclodextrin analytical column for both warfarin and hydroxywarfarin metabolites that were generated by the cytochrome P-450 enzymes during metabolism. The run time was long (exceeding 35 min) due to late elution times of the hydroxywarfarin metabolites and other endogenous plasma peaks that eluted even later in the chromatogram. The internal standard for this method was naproxen, now a common over-the-counter pain reliever. In this investigation, at least one plasma lot tested contained significant amounts of naproxen that would have affected quantitation of (R)- and (S)-warfarin in plasma.

This work changed the internal standard to oxybenzone (which is less likely to be present in the plasma at significant amounts) and used a column-switching device with a precolumn to separate and remove late eluting peaks before they could reach the analytical column. This reduced the run-time significantly, allowing (R)- and (S)-warfarin analysis within a 15-min run-time, without interference from late-eluting peaks in subsequent injections.

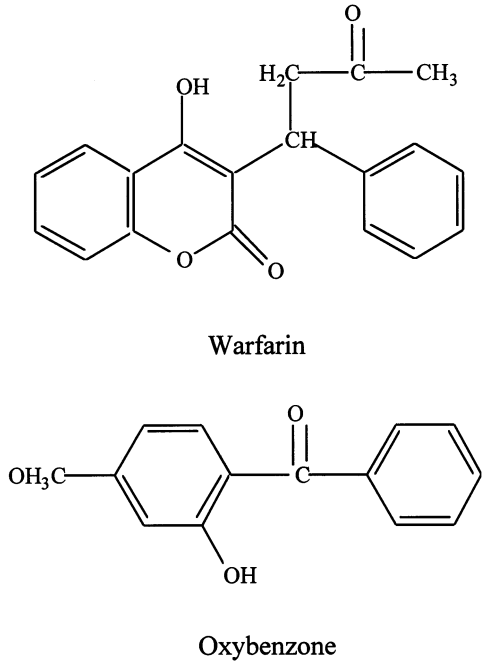

Fig. 1. Molecular structures of warfarin and oxybenzone (internal standard).

2. Experimental

2.1. Materials and reagents

Racemic warfarin was obtained from United States Pharmacopoeia (USP; Rockville, MD), (R)-(+)-warfarin and (S)-(−)-warfarin was from Ultrafine Chemicals/Gentest Corporation (Woburn, MA) and the internal standard (ISTD) oxybenzone was from Sigma (St Louis, MO). All organic solvents used were HPLC grade and purchased from Fisher (Fair Lawn, NJ) or Burdick and Jackson (Muskegon, MI). Deionized water was purified by a Milli-Q system (Millipore). Glacial acetic acid (HPLC grade) and 1.0 N sulfuric acid were purchased from Fisher, and triethylamine was purchased from Sigma. Control sodium ethylenediaminetetraacetic acid (EDTA) human plasma was obtained from Biochemed (Winchester, VA). Prosil®28 organosilane concentrate surface treating agent was obtained from PCR Incorporated (Gainesville, FL).

Single, individual stock solutions of (R)-(+)-warfarin and (S)-(−)-warfarin were prepared, and neat injections were made to determine elu-

tion order of the warfarin enantiomers. All subsequent work was completed using racemic warfarin. Warfarin was assumed racemic if the peak areas of (R)- and (S)-warfarin compared within $\pm 3.0\%$ when the racemic neat standard solution was injected onto the chromatograph.

Duplicate stock solutions of racemic warfarin were prepared from separate weightings for standards and quality control (QC) samples. Stock solutions and subsequent dilutions were prepared in methanol using glassware that had been treated with Prosil®28 organosilane agent, and were stored under refrigerated conditions. Calibration standard curves were prepared daily by adding 100 μl of tenfold concentrated solutions into 1.00 ml of blank human plasma to yield standards at levels of 12.5, 20.0, 100, 500, 1500, and 2500 ng/ml each of (R)- and (S)-warfarin in human plasma. Three levels of QC samples (25.0, 400 and 2000 ng/ml) were prepared, placed into glass storage vials, and stored at approximately -20°C.

2.2. Instrumentation

The HPLC system consisted of a Perkin Elmer Series 200 solvent delivery system (analytical pump), an SSI Series III Acuflow solvent delivery system (backflushing pump), a Rheodyne 7000 pneumatic valve-switching device, a Waters 715 autoinjector and an ABI Model 783A ultraviolet absorbance detector. The flow rate for each pump was 1.0 ml/min. The mobile phase was composed of acetonitrile:glacial acetic acid:triethylamine (1000:3:2.5, v/v/v). Detection was by absorbance at 320 nm. The analytical column was an Astec β-cyclodextrin column (250 × 4.6 mm, 5 μm), with an Astec β-cyclodextrin guard column (50 × 4.6 mm, 5 μm). The guard column remained in line with the analytical column for approximately the first 2 min of each injection, when it was taken out of line by the valve-switching device and backflushed to waste with mobile phase. At 12 min, the precolumn was placed back in line by the valve switching device. The total run time for each injection was 15 min.

Data was collected by a Waters Millennium 2020 Chromatography Manager data system, version 2.15.3 with data storage on a DEC-Alpha 4100 server running Alpha Open VMS® and IBM compatible client workstations running Windows 95®.

2.3. Extraction procedure

One milliliter of plasma was acidified by the addition of 700 μl of 1.0 N sulfuric acid. (R)-Warfarin, (S)-warfarin, and the internal standard were extracted from plasma with 5 ml of ethyl ether. The organic layer was removed and evaporated to dryness under a stream of nitrogen at 40°C. The sample was reconstituted in 200 μl of acetonitrile. Fifty microliters were then injected onto the HPLC system.

2.4. Data regression

Chromatographic data were collected and integrated by the Millennium 2020 Chromatography Manager system. Peak height ratios of (R)-warfarin/ISTD and (S)-warfarin/ISTD were calculated. The calibration curves were obtained by weighted ($1/\text{concentration}^2$) least-squares linear regression analysis. The equations of the calibration curves were then used to calculate the concentrations of (R)- and (S)-warfarin in the samples and QC samples by their peak height ratios.

3. Results and discussion

3.1. Separation

The molecular structures of warfarin and oxybenzone (internal standard) are shown in Fig. 1. (R)-warfarin, (S)-warfarin, and the internal standard were separated from each other, as shown in Fig. 2. The mean retention times for (R)-warfarin, (S)-warfarin, and the internal standard were 7.7, 6.9 and 4.0 min, respectively. Baseline or near baseline separation was achieved at all levels of (R)- and (S)-warfarin.

3.2. Specificity

Blank plasma from six separate lots of human plasma was tested for endogenous interferences.

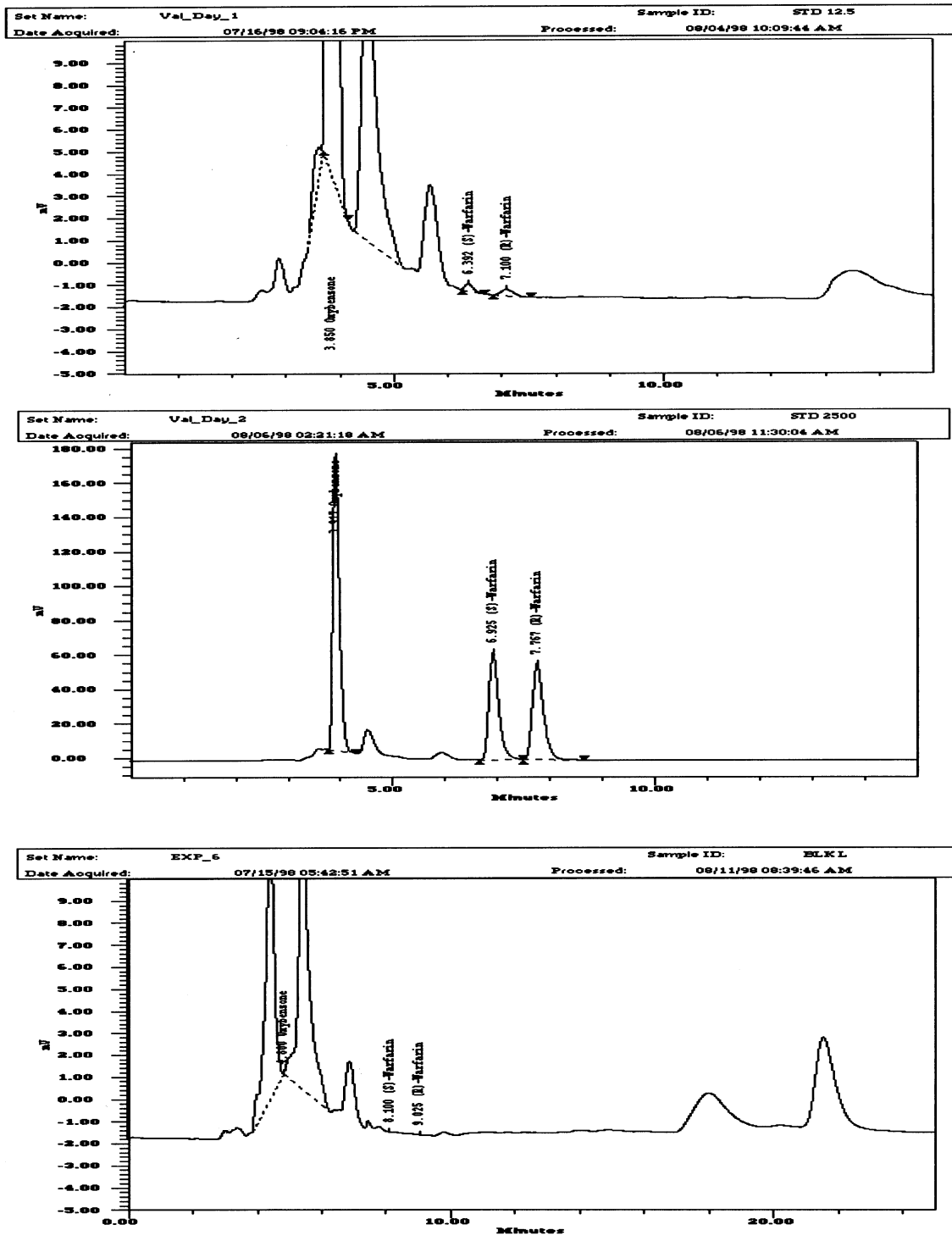

Fig. 2. Calibration standards containing 12.5 ng/ml (R)- and (S)-warfarin in human plasma (top), 2500 ng/ml (R)- and (S)-warfarin in human plasma (middle) and blank human plasma that is free of interference (bottom).

Table 1
Recovery of (R)-warfarin, (S)-warfarin and oxybenzone (internal standard) from human plasma

Quality control concentration (ng/ml)	(R)-Warfarin			(S)-Warfarin			Oxybenzone (internal standard)		
	Mean extract height ($n = 6$)	Mean pure height ($n = 6$)	Mean % recovery ($n = 6$)	Mean extract height ($n = 6$)	Mean pure height ($n = 6$)	Mean % recovery ($n = 6$)	Mean extract height ($n = 6$)	Mean pure height ($n = 6$)	Mean % recovery ($n = 6$)
25.0	536.1	639.7	83.8	614.2	708.3	86.7	158739.9	182781.4	86.9
400	8765.9	10761.7	81.5	9850.6	12062.6	81.7	163195.9	188575.6	86.5
2000	445638.2	53529.4	85.3	51363.4	60222.2	85.3	163611.8	192553.9	85.0
Overall recovery ($n = 18$)				83.5		84.6			86.1

Table 2
Calibration curve parameters and statistics: (R)- and (S)-warfarin in human plasma

Curve	(R)-Warfarin			(S)-Warfarin		
	Slope	y-Intercept	Correlation coefficient	Slope	y-Intercept	Correlation coefficient
1	1.310E-04	1.980E-06	0.9994	1.510E-04	−9.810E-05	0.9989
2	1.330E-04	−1.560E-04	0.9986	1.490E-04	−1.990E-05	0.9986
3	1.290E-04	−6.750E-05	0.9980	1.430E-04	1.760E-04	0.9977
Mean ($n=3$)	1.310E-04	−7.384E-05	0.9987	1.477E-04	1.933E-05	0.9984
S.D.	2.0000E-06			4.1633E-06		
R.S.D. (%)	1.5			2.8		

Of the batches tested, all were free of interference peaks in the internal standard region. There were minor peaks that were less than 5% of the internal standard peak height immediately preceding the internal standard region that did not affect quantitation. Four of the six plasma blanks tested were also free of interferences in both the (R)- and (S)-warfarin regions. One of the remaining two plasma blanks had a minor peak ($<25\%$ of the lower limit of quantitation) in the (S)-warfarin region. The sixth lot of plasma had a peak that was approximately the size of a 100-ng/ml peak in the (S)-warfarin region, and a peak that was the size of the lower limit of quantitation peak in the (R)-warfarin region. A representative chromatogram of a plasma blank is in Fig. 2.

The literature method [6] called for use of naproxen as an internal standard. However, this compound is a common over-the counter pain reliever, and was found in at least one blank plasma sample. It also had a late elution time, and a shorter analysis time was desired. Oxybenzone was selected as an alternate internal standard because it has an earlier elution time, excellent response, and extracts similarly to warfarin. There is some interference that immediately precedes the retention time of oxybenzone, but the oxybenzone response is so high that any endogenous interference is insignificant. Dioxybenzone was also evaluated as an internal standard, but was found to degrade to oxybenzone in the presence of warfarin.

Neat injections of racemic warfarin, (S)-warfarin, (R)-warfarin and oxybenzone were made to the chromatographic system. There were no extraneous peaks associated with the injection of the internal standard or racemic warfarin under the chromatographic conditions used. Injections of pure (S)-warfarin and (R)-warfarin were made at the beginning of work to determine the elution order of warfarin enantiomers, but were not used for quantitative purposes.

3.3. Absolute recoveries

Absolute recoveries were determined by comparing the peak heights of extracted QC samples with the peak heights of recovery standards (unextracted equivalents of extracted QC samples). The mean recoveries for (R)-warfarin, (S)-warfarin and the internal standard were 83.5, 84.6 and 86.1%, respectively (Table 1).

3.4. Method performance

Calibration curve parameters for (R)- and (S)-warfarin are in Table 2. Results were calculated using peak height ratios. Calibration curves for (R)- and (S)-warfarin in human plasma were linear using linear regression weighted $1/c^2$ in the concentration range from 12.5 to 2500 ng/ml, with correlation coefficients greater than or equal to 0.9977 for all curves. Precision for (R)- and (S)-warfarin in calibration standards, R.S.D. ($n=3$) was less than or equal to 3.6%, with deviations of mean values from nominal ($n=3$) less than or equal to 8.6% (Table 3). Precision for (R)- and (S)-warfarin in QC samples over the course of the

Table 3
Calibration curve data and statistics: (R)- and (S)-warfarin in human plasma

	Theoretical concentration (ng/ml)					
	12.5	20.0	100	500	1500	2500
(R)-Warfarin						
Mean ($n=3$)	12.7	19.8	92.9	514	1540	2527
S.D.	0.30	0.70	2.10	7.2	50.0	66.6
R.S.D. (%)	2.4	3.6	2.2	1.4	3.2	2.6
DMT (%)	1.6	−1.2	−7.1	2.8	2.7	1.1
(S)-Warfarin						
Mean ($n=3$)	12.6	20.1	91.4	515	1547	2533
S.D.	0.20	0.59	2.74	5.7	40.4	72.3
R.S.D. (%)	1.6	2.9	3.0	1.1	2.6	2.9
DMT[a] (%)	0.8	0.3	−8.6	3.1	3.1	1.3

[a] DMT, Deviation of mean value from nominal.

validation ($n=18$) was indicated by an R.S.D. of less than or equal to 10.9%, with deviations of mean values from nominal ($n=18$) less than or equal to 6.0% (Table 4). The lower limit of quantitation (LLOQ) for (R)- and (S)-warfarin in human plasma was set at 12.5 ng/ml. At the LLOQ, the R.S.D. ($n=6$) of the measured concentration was 5.8 and 4.7%, and the deviation of the mean of the measured concentrations from the nominal value was 1.5 and 7.2% for (R)-warfarin and (S)-warfarin, respectively.

3.5. Stability

QC samples containing 25.0, 400, and 2000 ng/ml (R)- and (S)-warfarin in plasma were subjected to three freeze/thaw cycles and to storage for 24 h at ambient temperature prior to extraction.

Three freeze/thaw cycles and ambient temperature storage of the QC samples for up to 24 h prior to analysis, appeared to have little effect on the quantitation of (R)- and (S)-warfarin in plasma. R.S.D. values remained below 2.8%, with deviations of mean values from theoretical within $\pm 10.3\%$. QC samples stored in a freezer set to maintain -10 to -30°C remained stable through the course of the validation (21 days). Calibration standards and duplicate QC samples were injected onto the HPLC after the extracts were allowed to stand at ambient temperature for at least 24 h prior to injection. Storing the extracted samples under these conditions prior to injection appeared to have no effect on quantitation of the standard curve or QC samples. Stock standard solutions remained stable for at least 37 days when stored in a refrigerator set to maintain 2–8°C. This conclusion is based on comparison of peak heights of a stock solution that had been stored for 37 days to those of a freshly prepared stock solution.

Table 4
Quality control sample data and statistics: (R)- and (S)-warfarin in human plasma

	Theoretical concentration (ng/ml)		
	25.0	400	2000
(R)-Warfarin			
Overall mean ($n=18$)	25.2	410	2112
S.D.	2.73	11.3	58.8
R.S.D. (%)	10.9	2.8	2.8
DMT[a] (%)	0.6	2.4	5.6
(S)-Warfarin			
Overall mean ($n=18$)	24.8	409	2119
S.D.	1.73	9.8	56.1
R.S.D. (%)	7.0	2.4	2.6
DMT[a] (%)	−0.7	2.3	6.0

[a] DMT, Deviation of mean value from nominal.

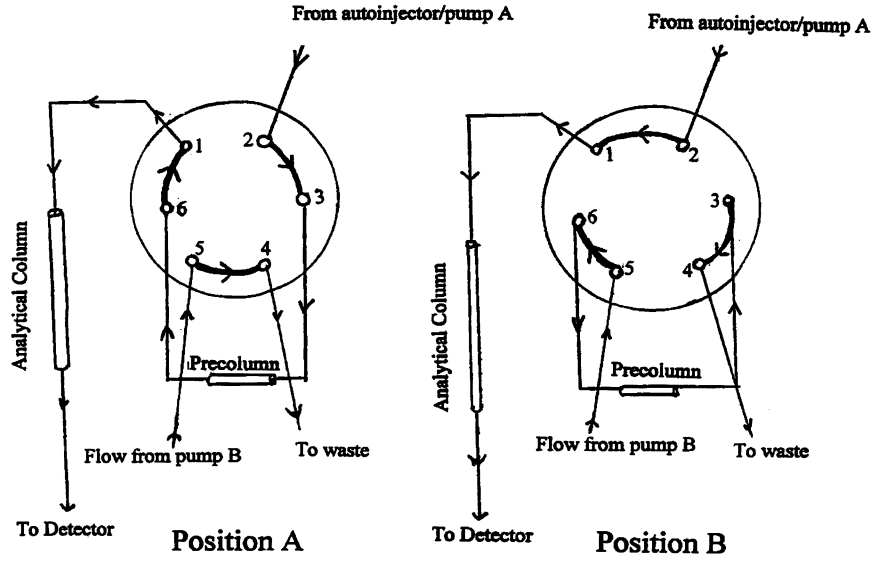

Fig. 3. Diagram of column switching device setup.

3.6. Use of the column switching device

To decrease run times, a Rheodyne 7000 switching valve was used as illustrated in Fig. 3. Position A placed the precolumn in line with the analytical column. Position B removed the precolumn from the line and backflushed to waste, while separation continued on the analytical column.

The sample was injected with the column switching device in position A. After (S)-warfarin and (R)-warfarin had eluted onto the analytical column, the precolumn was removed from the line and back-flushed with mobile phase at a flow rate of 1.0 ml/min. This prevented late-eluting peaks from reaching the analytical column, and decreased the run time by at least 10 min/injection from that used in the literature method [6].

3.7. Column stability

Two different Astec β-cyclodextrin analytical columns were used during the course of validation. Both yielded acceptable chromatography. On one occasion, a precolumn had to be replaced due to a deterioration in peak shape. The precolumn was removed, and an injection made to the analytical column to confirm that peak shape degradation was caused by the precolumn. A second precolumn placed on the system gave symmetrical peaks, but did not give peaks that were as sharp as the first precolumn, suggesting that some differences in chromatography may result from changing precolumns as well as the analytical column. This is consistent with the observations made in the literature method [6] regarding column variability.

4. Conclusions

The objective of this study was to validate an easy, rugged method for the determination of (R)- and (S)-warfarin in human plasma using a commercially available internal standard that would not normally be taken in conjunction with warfarin. A column-switching device was used in combination with the new internal standard and allowed the analysis time to be shortened to 15 min. This method allows as many as 96 injections to be made in a 24-h period, permitting rapid enantiomeric sample analysis in support of drug interaction studies. The extraction procedure and the chromatographic setup is simple, reliable and generates consistent responses.

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