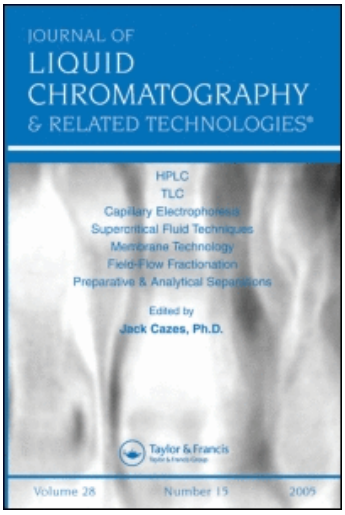

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VALIDATION OF A SEPARATION OF DIASTEREOMERS IN THE PHARMACEUTICAL INDUSTRY

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ABSTRACT

Method validation is an important step in any method development and has important implications in the pharmaceutical industry. Particular efforts should be directed towards the reproducibility, sensitivity and ruggedness of each method developed. In this paper, we report the validation of an HPLC method for the separation of the L-699,392 and its (S,R) diastereomer. This compound contains two chiral centers and a carboxyl functionality able to participate in a hydrogen bonding process. In order to obtain maximum sensitivity, the elution order of the two diastereomers was adjusted such that the minor diastereomer eluted before the major one. To achieve this elution order a nonpolar mobile phase consisting of methylene chloride and n-propanol containing quinine as a hydrogen bond acceptor was used. In order to optimize the separation, the influence of quinine concentration on the capacity and separation factor of the two diastereomers, influence of the polar modifier in the mobile phase and influence of the flow rate on the separation factor and system efficiency, were studied.

The method has been shown to be rugged giving base line separation of the two diastereomers with a limit of detection of 0.06% by weight for the minor diastereomer (S,R). The detector response of the (S,S) diastereomer was linear in the range 0.0005 to 1.1 mg/ml with an r^2 of 0.9996. The injection precision, linearity of the detector response and solution stability were also evaluated.

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INTRODUCTION

In the last decade, the emphasis on the production of enantiomerically pure drugs has increased greatly. Consequently, a large number of chromatographic methods able to separate enantiomers have been developed. Currently there are two main approaches for the direct separation of enantiomers: using either a chiral stationary phase or using an achiral stationary phase along with a chiral mobile phase [1,2,3]. The latter approach gives the analyst more flexibility in choosing different selectors for a particular enantiomeric separation.

A good alternative for the separation of enantiomers of acids and amines is to use optically active counter-ions dissolved in the mobile phase. The basis for resolution is the formation of diastereomeric complexes with different stability or distribution properties between the mobile phase and the stationary phase [4,5]. Two of the most effective selectors for ion-pairing the enantiomers of carboxylic acids are quinine and quinidine [6,7]. In order to enhance the strength of such interaction the selectors are usually dissolved in organic non-polar mobile phases.

Aryl and alkyl thioacetals of mercaptopropionic acid derivatives are potent receptor antagonists of leukotriene D_4 (LTD_4) and are being developed as therapeutic agents for bronchial diseases [8,9]. L-699,392 is a drug candidate with a potentially important role in the etiology of human asthma and other diseases suggesting that leukotriene antagonists will offer effective new therapy [10a, 10b].

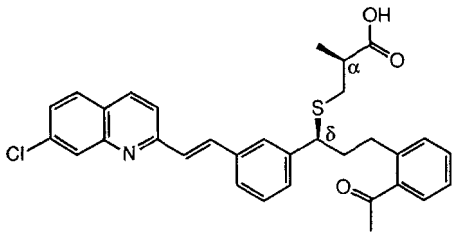

Figure 1. Chemical structure of L-699,392.

The structure of L-699,392 ((S,S) configuration) is presented in Figure 1. The compound has two asymmetric centers which are the carbon atoms in the α- and δ- positions relative to the carboxyl group. Consequently the compound is a diastereomer.

While the configuration of the δ-carbon is fixed through the synthesis, the α-carbon can undergo partial racemization during subsequent steps in the synthesis. Therefore the assessment of the optical purity of L-699,392 is essential.

Previous separation of the two diastereomers utilized a reversed phase HPLC system [11]. However, under those chromatographic conditions, the minor diastereomer (S,R) eluted after the major diastereomer (S,S). Such an elution pattern may have the drawback of poor detection limits, since it is possible that small amounts of (S,R) can be buried under the tail of the major diastereomer.

For this reason, a new HPLC method for the separation of the two diastereomers was developed. The stationary phase was a silica based diol and

the mobile phase was a mixture of methylene chloride and n-propanol which contained quinine as a selector. Under these experimental conditions the minor component, the (S,R) diastereomer elutes before the major component, the (S,S) diastereomer. This elution order, along with baseline separation, resulted in a better detection limit for the minor component.

EXPERIMENTAL

Sample Preparation

Samples were prepared in low actinic volumetric glassware by dissolving solid material into mobile phase to give a concentration of 0.5 mg/ml.

Stationary Phase

The stationary phase used in our experiments consisted of a 5 μm particle size silica based diol. The column (25 cm x 0.46 cm I.D.) was manufactured by E. Merck (Darmstadt, Germany) under the trade name Si-100 Lichrosphere DIOL.

Mobile Phase

Unless noted otherwise, the mobile phase consisted of a premixed solution of methylene chloride (E. Merck, Darmstadt, Germany) - n-propanol (E. Merck, Darmstadt, Germany) (99.5:0.5, v/v), containing 0.5 mM quinine and 0.5 mM acetic acid.

Chromatographic Conditions

The chromatography was performed on a Varian Vista 5500 single piston HPLC system (Varian, California, U.S.A.). Before injecting the samples, the column was equilibrated with the mobile phase until a steady baseline was obtained. The samples were injected into the chromatographic system through a 10 μl loop. Detection of the two diastereomers was performed by UV at 350 nm. The chromatograms were processed using PENelson Access*Chrom software (version 1.7) (PENelson, Cupertino, California).

RESULTS AND DISCUSSION

The structure of L-699,392 presented in Figure 1 shows that the compound has two chiral centers located at the carbons α and δ, relative to the carboxylic group. Therefore the compound is a diastereomer. The chiral center in the α position is fixed through the synthesis. The stereochemical structure of L-699,392 is presented in Figure 2. According to the Cahn-Ingold-Prelog convention, the configuration of the two carbons (C_α and C_δ) is (S,S). The structure of Figure 2 suggests that there are sufficient points of interaction to achieve the separation of the two diastereomers of L-699,392 using an achiral stationary phase.

Indeed, previous experiments showed that such a separation is possible, using a C-8 column along with reversed phase elution conditions [11]. The drawback of the separation was that under these experimental conditions the minor diastereomer eluted after the main diastereomer. The consequence of this elution order was a relatively poor detection limit for the minor diastereomer,

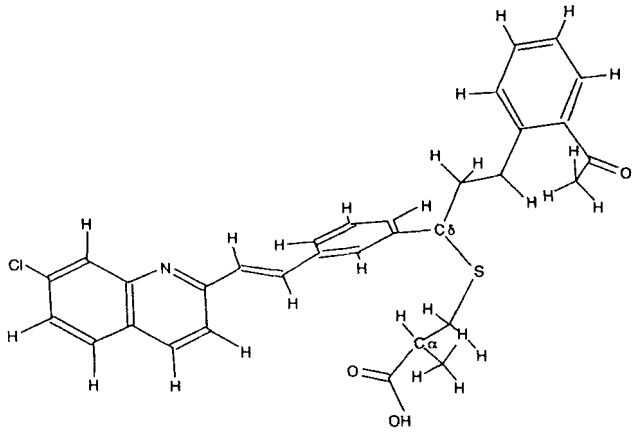

Figure 2. Stereochemical structure of L-699,392.

because the second peak eluted on the tail of the first. Under these circumstances, a new strategy was designed using a silica based diol column along with normal phase elution conditions, consisting of a mixture of methylene chloride - n-propanol (99.5:0.5, v/v). The diol column was selected because of its milder interaction with the analytes compared to the more interactive bare silica-gel stationary phase. In order to enhance the stereospecificity of the column, 0.5 mM quinine and 0.5 mM acetic acid were dissolved in the mobile phase. The addition of acetic acid was considered necessary due to its ability to compete with the solute for hydrogen bonding with quinine. Acetic acid will also compete in the adsorption to the stationary phase which has a limited adsorption capacity [4,5]. Such behavior will lead to lower values for the capacity factors of the two diastereomers without affecting the stereoselectivity of the system (data not shown). Under these conditions the chromatographic separation can be envisaged as a series of secondary chemical

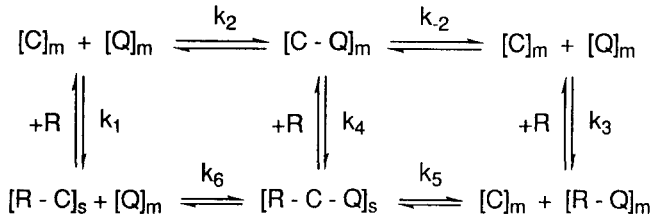

Figure 3. Secondary chemical equilibria for the ion-pairing separation.

equilibria (Figure 3). The subscript "m" relates to the processes occurring in the mobile phase, R is the stationary phase, the subscript "s" relates to the processes occurring in the stationary phase, [Q] is the concentration of quinine in the stationary/mobile phase and [C] is the concentration of the analytes in the stationary/mobile phase [12]. The process is very complicated and each step can be characterized by its own equilibrium constant.

The stereochemical structure of quinine is presented in Figure 4. The structure of Figure 4 shows that quinine is also a diastereomer.

In addition, the molecule contains a tertiary nitrogen (N-13), able to undergo hydrogen bonding with the carboxyl group of L-699,392 as a primary interaction. Other probable interactions between quinine and L-699,392 may occur via the aromatic regions of the two molecules (Figure 5).

The (S,R) diastereomer due to its stereochemical structure will lack such interactions. Therefore it can be suggested that the (S,S) diastereomer (L-699,392) will be stronger retained than the (S,R) diastereomer. A chromatogram of a mixture of the two diastereomers as well as a mixture of the two, enriched in L-699,392, showed the predicted elution order (Figure 6).

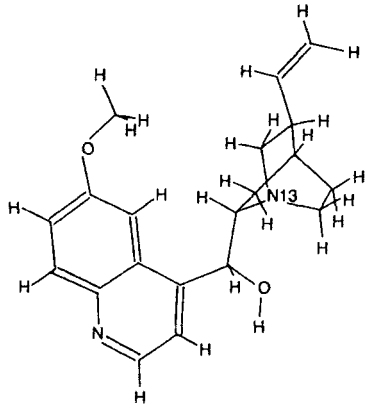

Figure 4. Stereochemical structure of quinine.

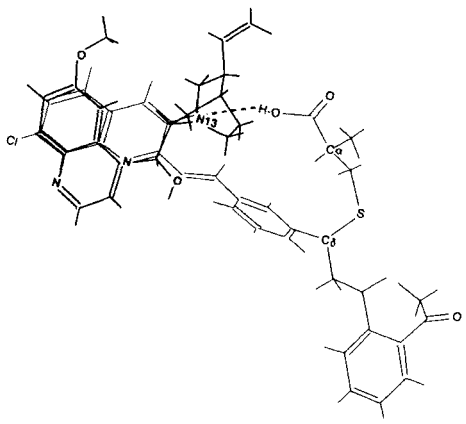

Figure 5. Probable interaction occurring between L-699,392 (S,S) diastereomer and quinine.

In order to validate the method, several parameters were varied to obtain the best conditions for the separation of the two diastereomers.

Influence quinine concentration on the separation factor (α) and capacity factor (k'). The amount of quinine in the mobile phase was varied

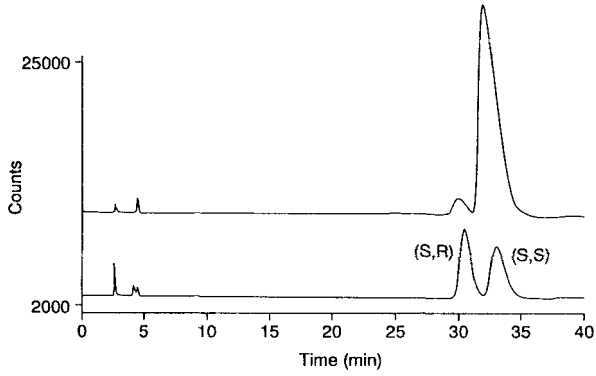

Figure 6. Chromatographic separation of (S,R) and (S,S) diastereomer. Chromatographic conditions: column - Lichrosphere DIOL (25 x 0.46 cm); mobile phase - methylene chloride/n-propanol (99.5:0.5, v/v) containing 0.5 mM quinine and 0.5 mM acetic acid; flow rate - 0.9 ml/min; detection - UV at 350 nm.

from zero to 1.5 mM. Upon changing the concentration, the column was first equilibrated in each mobile phase until a steady baseline was obtained. The variation of k' of the first eluted peak and of the separation factor with quinine concentration are shown in Figure 7 and Figure 8, respectively.

As the concentration of quinine increases, the value of k' steadily decreases. The separation factor, α, maximizes at a concentration of 0.25 mM quinine, followed by a decrease upon increasing the amount of quinine in the system (Figure 8).

Such a phenomenon may be due to an increase of quinine self-interaction at higher concentrations at the expense of quinine-analyte interaction. As a result, the analyte is less retained in the column and consequently the selectivity

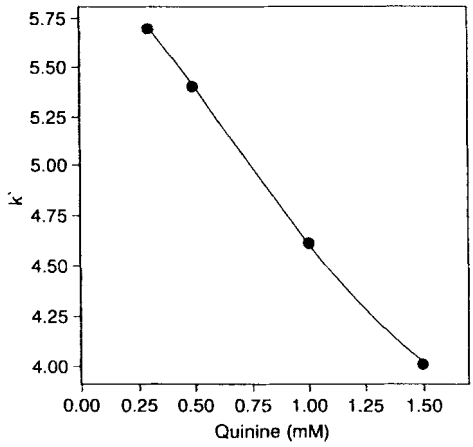

Figure 7. Influence of quinine concentration in the mobile phase on k' of the first eluted peak.

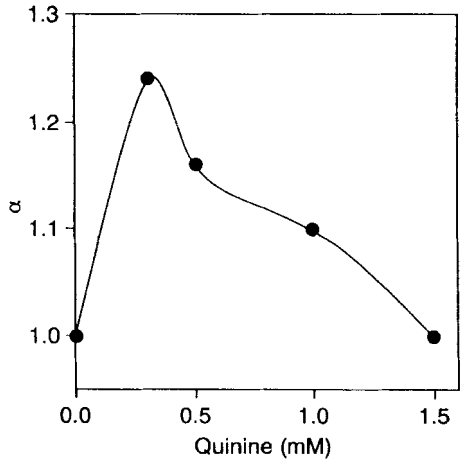

Figure 8. Influence of quinine concentration in the mobile phase on the separation factor of the two diastereomers.

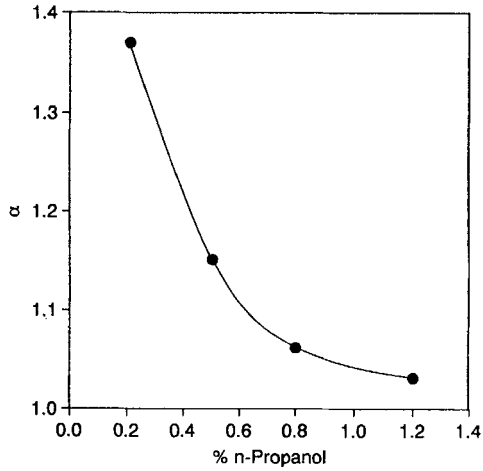

Figure 9. Influence of the percentage of n-propanol in the mobile phase on the separation factor of the two diastereomers.

diminishes as the concentration of the additive in the system increases. The optimum concentration of quinine with respect to both k' and α was considered to be 0.5 mM which produced baseline resolution. The next lower concentration could not be used due to excessive peak broadening, despite a better α value for the two diastereomers. Peak broadening will lead to a deterioration of the detection limit for the minor diastereomer (S,R).

Influence of n-propanol on α. To ascertain the optimum amount of the polar component in the mobile phase, the concentration of n-propanol was varied from 0.2 to 1.2% (v/v) for a constant quinine concentration and the resulting α values were determined (Figure 9).

Upon increasing the amount of n-propanol in the mobile phase, the two diastereomers become solvated, and as a result the interaction with quinine diminishes. Consequently, α decreases. The optimum concentration of n-propanol was considered to be 0.5% (v/v). Despite the fact that lower concentrations of n-propanol in the mobile phase produced higher α values, excessive peak broadening was observed.

Influence of the flow rate on α values and system efficiency. Flow rate has a very important influence on a particular separation in an HPLC system. Lower flow rates lead to longer retention times and consequently slower separations. Therefore the choice of flow rate often requires a compromise between separation speed and efficiency [13].

In our case the flow rate was varied from 0.2 ml/min to 1.2 ml/min and its influence on HETP (high equivalent of a theoretical plate) and α was studied. As the flow rate of the mobile phase was increased, HETP decreased (consequently the efficiency of the system increased) up to a value corresponding to a flow rate of 0.9 ml/min. Beyond this value, HETP increased leading to a less efficient system. The van Deemter plot is shown in Figure 10.

At the same time α decreases steadily with the increase of the flow rate (Figure 11).

As a result a flow rate of 0.9 ml/min was selected. Lower flow rates produced higher α values, at the expense of poor peak efficiency.

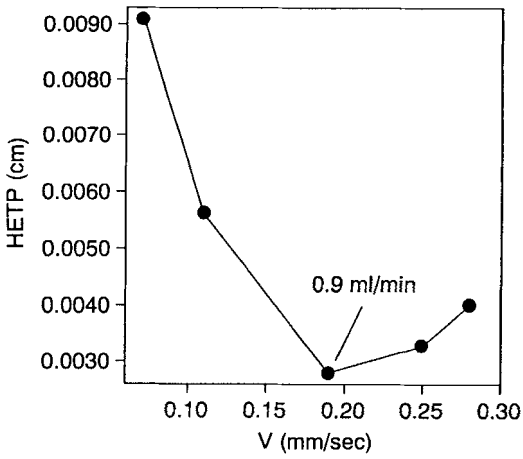

Figure 10. Van Deemter plot for the first eluted peak. For chromatographic conditions see Fig. 6, except the value for the flow rate.

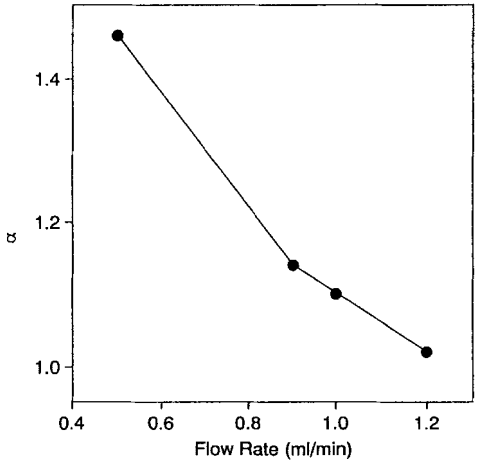

Figure 11. Influence of the flow rate on the separation factor of the two diastereomers. For the chromatographic conditions see Fig. 6 except the value for the flow rate.

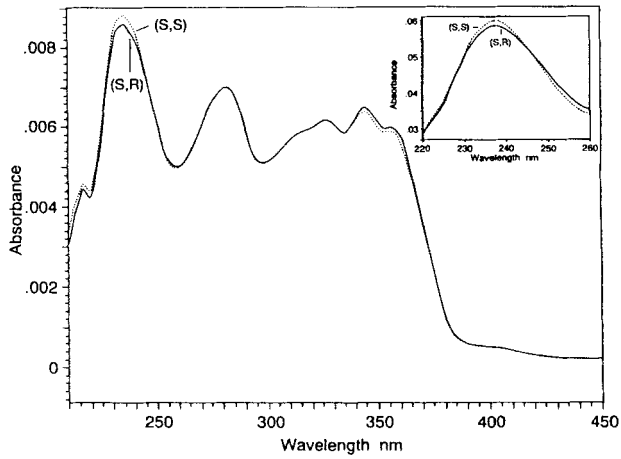

Figure 12. UV spectra of the complex of quinine with (S,S) and (S,R) diastereomer. The spectra were acquired using a Waters photo diode array.

With the separation parameters optimized, the next step in the validation was to study the linear dynamic range of the detector, limits of detection and quantitation, injection to injection reproducibility, retention time reproducibility and the solution stability of the two diastereomers.

Study of the linear dynamic range of the (S,S) and (S,R) diastereomer.

Upon interaction of (S,S) and (S,R) diastereomer with quinine, new diastereomeric complexes were formed. In such instances there is a possibility that the two complex species may have different extinction coefficients or UV spectra. Therefore, the UV spectra of the two peaks corresponding to the two diastereomers were taken, using a Waters 990 photo diode array detector (Milford, MA). The two spectra (Figure 12) showed small differences in

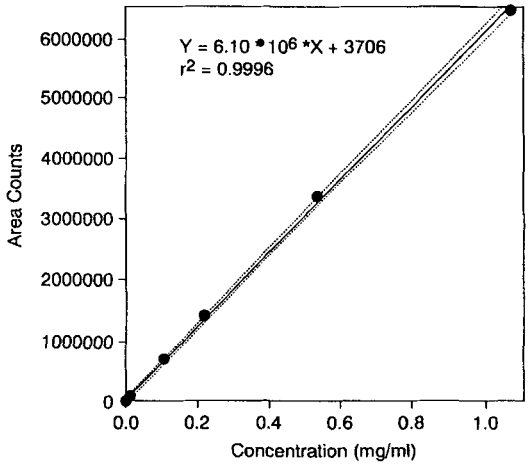

Figure 13. Calibration curve for the (S,S) diastereomer at 350 nm.

absorbance in the area of 238 nm and 350 nm with an isosbestic point at 245 nm. Therefore, the linear dynamic range of the two diastereomers was studied individually. Since quinine absorbance extends up to 300 nm, a monitoring wave length of 350 nm was selected during the chromatography of the two diastereomers.

For the (S,S) diastereomer the range of concentration studied was between 0.0005 mg/ml to 1.1 mg/ml and for the (S,R) diastereomer it was between 0.0001 to 0.01 mg/ml. The results are presented in Figures 13 and 14. A straight line was obtained in both cases with an $r^2 = 0.9996$ and 0.9992 respectively.

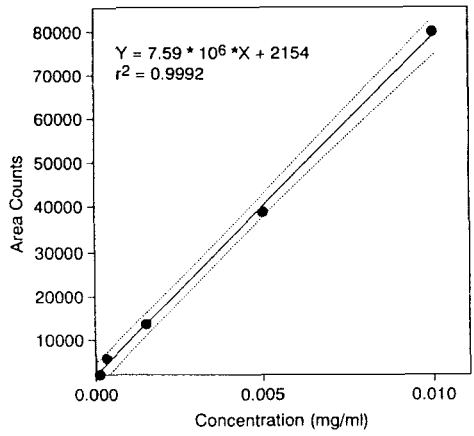

Figure 14. Calibration curve for the (S,R) diastereomer at 350 nm.

The optimum concentration for the (S,S) diastereomer was chosen to be 0.5 mg/ml. Above this concentration overloading effects occurred with a loss in resolution.

Study of the limits of quantitation and detection for the (S,R) diastereomer. The limit of quantitation was determined by injecting into the system increasing amounts of the minor diastereomer (S,R). Each concentration was repeated three times. Plotting percentage relative standard deviation (% RSD) versus the amount injected, a curve was obtained with an inflection point which corresponds to the limit of quantitation. The results are presented in Figure 15, and the limit of quantitation is 0.0003 mg/ml.

This corresponds to 0.1% (S,R) diastereomer relative to an injected concentration of 0.5 mg/ml L-699,392 (the major diastereomer). The detection

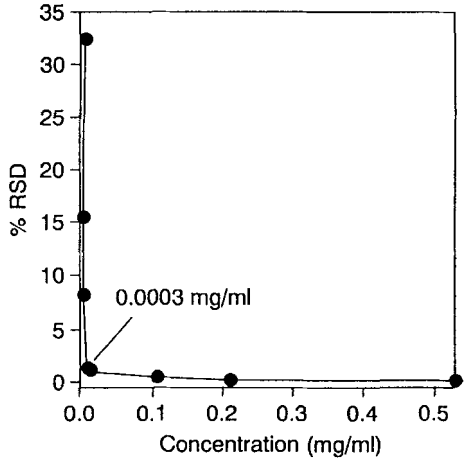

Figure 15. Limit of quantitation for the (S,R) diastereomer at 350 nm.

limit for the (S,R) diastereomer was determined to be at 0.06% of the major diastereomer at a signal-to-noise ratio of 3:1.

Study of injection precision and retention time reproducibility. The reproducibility of the system was established by performing replicate injections of the same solution containing a mixture of the (S,S) and (S,R) diastereomers (99.7:0.3, w/w). The area counts and relative retention times (RRT) were recorded for each injection and the %RSD were calculated. The data are presented in Table 1.

The results show a value of 0.3% RSD for the (S,S) diastereomer and 3.6% RSD for the (S,R) diastereomer. The increased percentage RSD for the minor diastereomer is due to the low level at which it was determined. The

TABLE 1

Assay Reproducibility as Function of Injection Number and Relative Retention Time (RRT) for the (S,R) Diastereomer

Injection number	Area Counts (S,S)	(S,R)	RRT (S,R)
1	3381063	8710	0.93
2	3367519	9315	0.92
3	3393540	9050	0.93
4	3378140	8860	0.93
5	3372123	9530	0.92
6	3369654	8768	0.93
Mean	3377006	9038	0.93
%RSD	0.3	3.6	0.60

Table 2

Solution Stability for the Two Diastereomers

Time (hrs)	% Found (S,S)	(R,S)
0	99.40	0.60
2	99.40	0.60
4	99.40	0.60
8	99.40	0.60
16	99.40	0.60
24	99.50	0.50

%RSD for the relative retention time of the minor diastereomer was determined to be 0.6%.

Study of the solution stability of the (S,S) and (S,R) diastereomer. In order to determine if there was any conversion of the (S,S) to (S,R) configuration, a sample containing 99.4% (S,S) and 0.6% (S,R) diastereomer was repeatedly injected over a 24 hour period.

The results of Table 2 show that over the period of time studied no such conversion occurred. This method is also capable of separating the other pair of diastereomers ((R,R) and (S,R)). A similar stability study was performed in order to investigate wether any racemization may occur at the δ carbon. Over the same period of time no racemization occurred (data not shown).

CONCLUSIONS

An ion-pair chromatographic system with quinine in the mobile phase was successfully applied to the separation of the two diastereomers of L-699,392. Under the chromatographic conditions the two diastereomers of interest were baseline resolved with the minor diastereomer eluting first. Quantitation of (S,R) diastereomer at low levels was easily achieved. The method was rugged, reproducible and linear over a wide concentration range.

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