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# Determination of citalopram enantiomers in human plasma by liquid chromatographic separation on a Chiral-AGP column

Dan Haupt

*Analytical Pharmaceutical Chemistry, Uppsala University, Biomedical Centre, P.O. Box 574, S-751 23 Uppsala, Sweden*

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**Abstract**

A liquid chromatographic method for the quantitative analysis of *S*-(+)- and *R*-(−)-citalopram in human plasma has been developed and validated. The enantiomers of citalopram and the internal standard, *R*-(+)-propranolol, were extracted from alkaline plasma with 2% *n*-butanol in *n*-hexane. After a clean-up step, the organic phase was evaporated and the residues dissolved in 50–100 μl of 0.001 *M* HCl. The separation was performed on a Chiral-AGP column with 3.0 m*M* N-dodecyl-N,N-dimethylammonio-3-propanesulfonate and 10 m*M* hexanoic acid in phosphate buffer pH 6.5 as the mobile phase. The limit of detection was estimated to be 1 ng/ml ($S/N \approx 3$) for each enantiomer monitoring UV absorption at 240 nm. In the range studied, 2.31–191 ng/ml, the recoveries were quantitative and the coefficients of variations were between 2.47% and 11.5%.

*Keywords:* Enantiomer separation; Citalopram

## 1. Introduction

Major depressive disorder is a very common medical problem affecting about 10% of humanity [1]. Citalopram, a potent and highly selective serotonin reuptake inhibitor, is an interesting alternative in the treatment of depressive patients as it appears to have few and mild side effects compared to the classical antidepressants [1–3]. Citalopram, marketed as a racemate, is mainly metabolized by N-demethylation to form desmethylcitalopram and didesmethylcitalopram [4]. Other metabolites found in plasma and urine are the N-oxide of citalopram and different propionic acid metabolites [5]. Recently, a report stated that the pharmacological effect of citalopram is related mainly to *S*-(+)-citalopram and to some extent in *S*-(+)-desmethylcitalopram [6]. In the serotonin uptake test, eudismic ratios of 167 and 6.6 were obtained for the enantiomers of citalopram and desmethylcitalopram, respectively. These facts imply that the presence of the distomer [*R*-(−)-citalopram] in the racemate probably does not contribute to the antidepressive effect of citalopram [6].

The study of citalopram bioavailability requires a highly sensitive analytical method because of the low plasma levels (15–620 n*M* for the racemate) resulting from therapeutic doses [2,7]. A normal-phase high-performance liquid chromatography (HPLC) method has been used for the quantitative analysis of the enantiomers of citalopram and two of its metabolites in plasma of depressive patients treated with citalopram [8]. The method comprised a derivatisation step before the chiral resolution on a Chiralcel OD column. A faster method, using Cyclobond columns with aqueous phases, was used for the analysis of the underivatized enantiomers of citalop-

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ram and its demethylated metabolites in plasma samples obtained from depressive patients [9]. Interestingly, the concentrations of the distomer, *R*-(−)-citalopram, were found to be higher than the concentrations of the eutomer, *S*-(+)-citalopram, with a mean *S*/*R* ratio of 0.56. As yet, the relationship between citalopram concentration and therapeutic effect is elusive and the importance of interindividual differences in plasma levels of its enantiomers cannot be neglected.

A new method for the quantitative analysis of the enantiomers of citalopram in human plasma is presented. The method involves a liquid–liquid extraction procedure followed by chiral resolution on a Chiral-AGP column. The method has been validated with respect to recovery, linearity, precision and limits of detection and quantitation.

## 2. Materials and methods

### 2.1. Chemicals

*S*-(+)-Citalopram oxalate and *R*-(−)-citalopram oxalate were kindly supplied by Lundbeck (Copenhagen, Denmark). N-Decyl-N,N-dimethylammonio-3-propanesulfonate (SB-10), N-dodecyl-N,N-dimethylammonio-3-propanesulfonate (SB-12), N-tetradecyl-N, N-dimethylammonio-3-propanesulfonate (SB-14), N-hexadecyl-N,N-dimethylammonio-3-propanesulfonate (SB-16) and *R*-(+)-propranolol hydrochloride were purchased from Sigma (St. Louis, MO, USA). Hexanoic acid was from Aldrich Chemie (Steinheim, Germany). Dodecylethyldimethylammonium bromide (DDEDMA) was from Fluka (Buchs, Switzerland). Orthophosphoric acid (p.a.), hydrochloric acid (p.a.) and methylene chloride (p.a.) were from Merck (Darmstadt, Germany). Sodium hydroxide was from EKA Nobel (Sweden). A stock solution of purified hexanoic acid was prepared as follows; 120 ml of a 1 *M* sodium hydroxide solution was mixed with 4.70 g of hexanoic acid and 50 ml of methylene chloride. The mixture was shaken for 5 min before the organic phase was discarded. The aqueous phase was transferred to another bottle where it was degassed until no organic smell could be discerned.

All mobile phases were prepared with deionized water, purified with a Milli-Q purification system (Millipore, Bedford, MO, USA).

All glassware was silanized with Sigmacote (Sigma) before use.

### 2.2. Apparatus

The chromatographic system consisted of two LKB 2150 HPLC pumps (Pharmacia, Uppsala, Sweden), a LKB 2152 HPLC controller and a LKB 2152-400 high-pressure mixer. The injector was a Rheodyne 7725i equipped with a 40-μl loop. The UV absorbance was monitored at 240 nm using a Model 3100 LDC analytical spectrophotometer. The recorder was a Kipp and Zonen BD 112 (Kipp and Zonen, Delft, Netherlands). The separations were performed on a Chiral-AGP column (100×4.0 mm I.D.) from ChromTech AB (Norsborg, Sweden). The water bath used for thermostating the column was HETO Type 02 PT 923 TC (Birkerød, Denmark). The pH measurements were made with an AG 9100 Metrohm 632 pH meter (Herisau, Switzerland) equipped with a Type 1014 glass pH electrode.

### 2.3. Chromatographic conditions

The standard conditions used during the chromatography were a flow-rate of 0.90 ml/min and a column temperature of 32.7°C. The column was thermostated by pumping water from the water bath through a glass-jacket mounted on the column. The mobile phases were prepared by making a phosphate buffer from 1 *M* phosphoric acid and 1 *M* sodium hydroxide to which different modifiers were added before diluting with deionized water to a final ionic strength of 0.1 *M*. The pH was then adjusted to the desired level by adding the proper amount of 1 *M* sodium hydroxide or 1 *M* phosphoric acid.

### 2.4. Standards

Stock solutions of *S*-(+)- and *R*-(−)-citalopram oxalate and *R*-(+)-propranolol hydrochloride were made in deionized water at a concentration of 0.46–1.3 μg/ml. The stock solution of 0.33 *M* DDEDMA was also made in deionized water. Working solutions were prepared by mixing the appropriate volume of the stock solutions and diluting with deionized water

to the desired concentration. All solutions were stored in a refrigerator at −4°C until use.

### 2.5. Extraction procedure

To 2 ml of spiked plasma were added 175 $\mu$l (1.32 $\mu$g/ml) of internal standard [*R*-(+)-propranolol], 150 $\mu$l of 1 *M* sodium hydroxide, 40 $\mu$l of 0.33 *M* DDEDMA and 10 ml of 2% *n*-butanol–hexane (2:100, v/v). The mixture was shaken for 20 min and centrifuged at 1800 *g* for 4 min before freezing the aqueous phase with dry-ice. The organic phase was transferred into another glass tube containing 1.5 ml of 0.1 *M* hydrochloric acid. The solution was shaken for 20 min followed by centrifugation at 1800 *g* for 4 min. The aqueous phase was frozen and the organic phase discarded. To the aqueous phase was added 0.5 ml of 1 *M* sodium hydroxide and 4 ml of 2% *n*-butanol–hexane. The mixture was shaken for 20 min and centrifuged at 1800 *g* for 4 min before freezing the aqueous phase with dry ice. The organic phase was transferred to another glass tube and evaporated to dryness at 90°C under a stream of air. Finally, the compounds were dissolved in 50–100 $\mu$l of 0.001 *M* HCl by vortex-mixing for 30 s.

## 3. Results and discussion

### 3.1. Optimization of the chiral separation

The enantioselective retention of solutes on Chiral-AGP is generally optimized by changing pH or addition of charged and/or uncharged modifiers [10]. Usually both the retention and the enantioselectivity decrease with increasing concentration of uncharged modifier in the mobile phase. Previously, an uncharged micellar agent, Tween 20, has been used as an alternative to the commonly used organic modifiers (e.g. methanol, propanol) for regulating enantioseparation of hydrophobic enantiomers on $\alpha_1$-acid glycoprotein columns [11–14]. The results obtained indicate several interesting properties of the micellar systems used e.g. reduced retention with maintained enantioselectivity for hydrophobic enantiomers.

On a Chiral-AGP column with a mobile phase of phosphate buffer pH 6.5 and 1.6 m*M* of the zwitterionic detergent SB-14 capacity factors of 5.8 were

Table 1
Influence of organic modifier on chiral resolution

Separation column: Chiral-AGP, 100×4.0 mm I.D.;
Mobile phase: phosphate buffer pH 6.5 and different additives
Solute: *S*-(+)- and *R*-(−)-citalopram
temperature: 25.0°C

| Additive | $k'_R$ | $k'_S$ | $\alpha$ | $R_S$ |
|---|---|---|---|---|
| 1.6 m*M* SB-14 | 5.8 | 5.8 | 1.0 | - |
| 1.6 m*M* SB-14+ 0.9 m*M* hexanoic acid | 6.1 | 6.2 | 1.02 | - |
| 1.6 m*M* SB-14+ 10 m*M* hexanoic acid | 7.3 | 11 | 1.53 | 2.37 |
| 1.6 m*M* SB-16+ 10 m*M* hexanoic acid | 9.4 | 14 | 1.52 | 2.11 |
| 1.6 m*M* SB-12+ 10 m*M* hexanoic acid | 5.7 | 8.7 | 1.53 | 2.14 |
| 3.2 m*M* SB-10+ 10 m*M* hexanoic acid | 8.6 | 19 | 2.16 | 6.64 |

$\alpha = k'_S/k'_R$, $R_S = 2(t_{R2} - t_{R1})/(W_{t2} + W_{t1})$.

obtained for both enantiomers of citalopram, (Table 1 and Fig. 1). Addition of 10 m*M* of hexanoic acid resulted in a drastic improvement in enantioselectivity and resolution. This is due to the strong increase in retention for the second eluted enantiomer, *S*-(+)-citalopram. Unfortunately, also the retention time for the internal standard, *R*-(+)-propranolol, could be expected to increase and the time for the chromatographic run became unacceptable long. Previous studies have shown that an efficient decrease in retention can be obtained by choosing the proper concentration and type of detergent as mobile phase additive [13,15]. Several different zwitterionic detergents with the same functional groups were therefore tested. Exchange of SB-14 for the more hydrophobic detergent SB-16 increased the retention for both enantiomers while the more hydrophilic detergent SB-12 decreased the retention, Table 1. The mechanism of retention seems at first sight related to the hydrophobicity of the detergent or the critical micellar concentration (CMC) of the detergent. According to the literature [16] the CMC values are; 27 m*M* (SB-10), 2.0 m*M* (SB-12), 0.20 m*M* (SB-14) and 0.015 m*M* (SB-16). This means that only SB-14 and SB-16 will form micelles in the mobile phases used. Berry and Weber studied the effects of SB-12 on the retention of a group substituted benzene compounds on an RP-18 column and

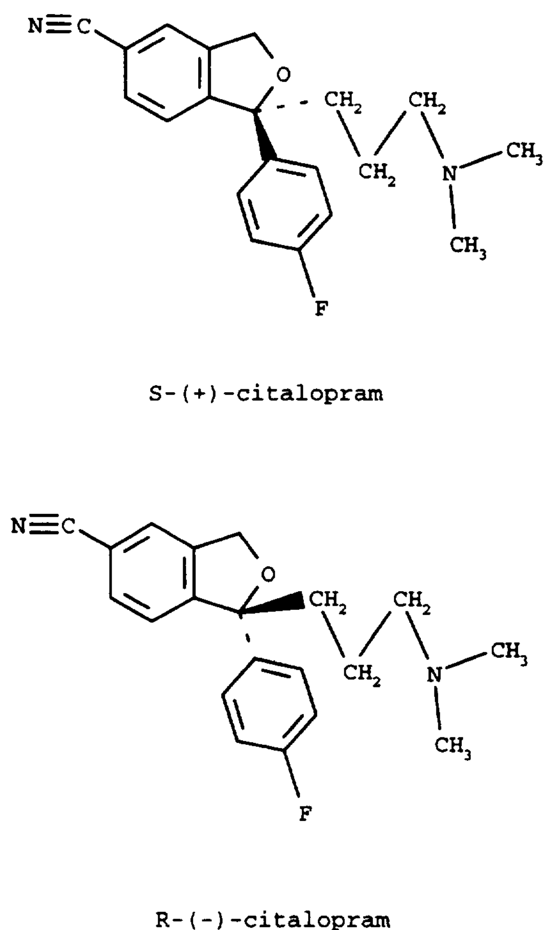


Fig. 1. Chemical structure of citalopram.

concluded that the partitioning of the solutes in SB-12 micelles is mainly governed by hydrophobic interactions [17]. Accordingly, an increase in the chain length (hydrophobicity) of the detergent would decrease the retention for the enantiomers if they are distributed to the micelles. However, the retention is not solely governed by the micellar concentration. The detergent SB-12, used in concentration below CMC is very efficient in reducing retention times for the enantiomers of citalopram. This fact shows that one of the mechanisms for retention is a competition between the enantiomer(s) and the detergents for one or several sites on the $\alpha_1$-acid glycoprotein. Furthermore, the monomers of the more hydrophobic detergents, i.e. SB-14 and SB-16, are probably more efficient in decreasing the retention times for the enantiomers but are also present in much lower concentrations due to the micellization process, cf. CMC-values. In summary, the decrease in retention of the solutes obtained by distribution to the micelles, if any, is probably counteracted by the lower concentrations of monomers available. However, more data is necessary to confirm this hypothesis.

Another zwitterionic detergent, SB-10, was also tested and once again the complexity of the chiral recognition mechanism on Chiral-AGP was demonstrated. The increase in $k'$ for *R*-(−)-citalopram may be explained by a lower competitive effect compared to SB-12, but the increase in $k'$ of *S*-(+)-citalopram as well as the increase in $R_S$ to 6.64 indicates an increase in the affinity of $\alpha_1$-acid glycoprotein for this compound. An explanation for this effect may be allosteric interaction as discussed by Wainer and Noctor [18].

In conclusion, the mobile phase comprising the zwitterionic detergent SB-12 (1.6 m*M*) and hexanoic acid (10 m*M*) in phosphate buffer pH 6.5 promoted a complete resolution of (*R*,*S*)-citalopram with reasonable retention times for the solutes of interest. To attain highest possible throughput and gain highest possible detectability, the SB-12 concentration was finally increased to 3 m*M* before starting the bioanalysis of citalopram.

### 3.2. Absolute recovery

Initially, a quaternary ammonium component, DDEDMA, was used in the extraction procedure to prevent adsorption of the solutes to glassware and/or plasma proteins. In each extraction step (see extraction procedure) 40 μl of 0.33 *M* DDEDMA was added. Furthermore, the residues were dissolved in 5 m*M* DDEDMA in 0.01 *M* HCl before injection onto the Chiral-AGP column. Unfortunately, a fast impairment of the column performance was observed when using DDEDMA. Additional experiments showed that the removal of the quaternary component from all extraction steps except the first one was possible without influencing the recoveries.

Absolute recoveries were studied for two concentrations of each enantiomer and the concentration used for the internal standard. A 2-ml volume of blank, pooled plasma was spiked with the two enantiomers and the internal standard and thereafter

extracted as described in Section 2.5. The obtained peak areas were then compared with the peak areas of solutions directly injected into the chromatographic system (Table 2). The mean recovery from plasma was around 100% for the two enantiomers at the higher concentrations and 120% and 109% for the *R*-(−)- and *S*-(+)-enantiomer, respectively, at the lower concentrations.

### *3.3. Linearity of the method*

In order to check the linearity of the method, plasma samples spiked with increasing amounts of the enantiomers (9–190 ng/ml) and a constant amount of the internal standard were analyzed. The peak area ratios were calculated and the data obtained were analyzed by linear regression. Calibration curves constructed over the concentration ranges for *R*-(−)-citalopram and *S*-(+)-citalopram showed good linearity. The equations of the calibration curves were:

*R*(−)citalopram,

$$y = 0.00738 + 0.0131x, r = 0.999$$

*S*(+)citalopram,

$$y = -0.00318 + 0.0156x, r = 0.999$$

where $y$ is the ratio between the peak area of the enantiomer and that of the internal standard and $x$ is the amount of the enantiomer in ng/ml.

### *3.4. The limit of quantification and limit of detection*

Blank plasma samples were extracted as described above and the residues were injected on the chromatographic system. The blank chromatograms were adequately clean at the retentions of the two enantiomers. Aarons et al. [19] defined the limit of quantification (LOQ) as the level having a predefined coefficient of variation (C.V.) of 20%. The LOQ validated in this way should have a calculated value within 20% of nominal. Further, any interfering peak should have an area 20% or less than that of the LOQ [19]. Another definition of the LOQ has been discussed by Buick et al. [20] which defined the LOQ as the lowest concentration of analyte that can be measured with acceptable precision and accuracy by the method. The definition of acceptable is dependent on the aims and objectivity of the work which the method is supporting and should be decided by the analyst [20].

Our definition of the LOQ was that accuracy, defined as 100(obtained conc./added conc.) should fall within ±20% of nominal and that the C.V. calculated on absolute recovery should fall within 20%. According to these criteria, the LOQ for the *R*-(−)-enantiomer was estimated to be 2.31 ng/ml and the LOQ for *S*-(+)-citalopram was 2.55 ng/ml (see Table 2). No interfering peak >20% of the LOQ could be distinguished on the chromatograms for these enantiomers (Fig. 2).

The limit of detection was estimated to be approximately 1 ng/ml ($S/N \approx 3$) for each enantiomer, using 2 ml of samples.

Table 2
Absolute recovery

| Substance | Added (ng/ml) | Recovery ($n=7$) (mean±S.D.) (%) | C.V. (%) |
|---|---|---|---|
| *R*-(−)-Citalopram | 2.31 | 120±19.7 | 16.5 |
| | 118 | 99.0±5.81 | 5.86 |
| *S*-(+)-Citalopram | 2.55 | 109±16.5 | 15.1 |
| | 130 | 100±7.60 | 7.60 |
| *R*-(+)-Propranolol | 115 | 103±5.33 | 5.20 |

### *3.5. Within-day reproducibility and day-to-day reproducibility*

Within-day reproducibility was tested on 2 ml of pooled plasma spiked with 115 ng/ml of internal

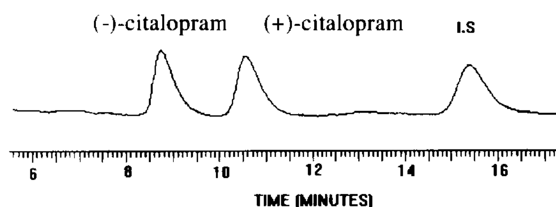


Fig. 2. Separation of *R*-(−) and *S*-(+)-citalopram isolated from human plasma. Plasma sample spiked with 63.6 ng/ml and 70.0 ng/ml of *R*-(−)- and *S*-(+)-citalopram, respectively. Mobile phase: 10 m*M* hexanoic acid and 3.0 m*M* SB-12 in phosphate buffer pH 6.5. Separation column: Chiral-AGP, 100×4.0 mm. Flow-rate: 0.90 ml/min. Temperature: 32.7°C.

Table 3
Within-day reproducibility and accuracy

| Added (ng/ml) | Found (mean±S.D., $n=7$) (ng/ml) | C.V. (%) | Accuracy[a] (%) |
|---|---|---|---|
| *(−)-Citalopram* | | | |
| 2.31 | 2.67±0.19 | 7.18 | 116 |
| 4.62 | 4.89±0.56 | 11.5 | 105 |
| 9.25 | 9.34±0.74 | 7.96 | 101 |
| 63.6 | 66.8±2.7 | 4.00 | 105 |
| 118 | 123±3.9 | 3.19 | 104 |
| 173 | 183±5.8 | 3.15 | 106 |
| *(+)-Citalopram* | | | |
| 2.55 | 2.60±0.18 | 6.74 | 102 |
| 5.10 | 4.66±0.20 | 4.22 | 91.4 |
| 10.2 | 9.64±0.56 | 5.79 | 94.5 |
| 70 | 71.4±2.7 | 3.73 | 102 |
| 130 | 134±5.8 | 4.32 | 103 |
| 191 | 182±7.6 | 4.20 | 95.3 |

[a] Accuracy defined as: 100×[Obtained/Added].

standard and 2.31–173 ng/ml and 2.55–191 ng/ml of the *R*-(−)- and *S*-(+)-enantiomer, respectively, Table 3. Coefficients of variation (C.V.) obtained on the same day were ≤11.5% for the *R*-(−)-enantiomer and ≤6.74% for the *S*-(+)-enantiomer.

Day-to-day reproducibility was tested over a period of 13 days (Table 4). The plasma concentrations were 9.25 and 118 ng/ml for the *R*-(−)-enantiomer and 10.2 and 130 ng/ml for the *S*-(+)-enantiomer. The day-to-day coefficient of variation was between 3.19% and 9.42% for the *R*-(−)-enantiomer and between 2.47% and 10.0% for the *S*-(+)-enantiomer. No concentration or time related trend in accuracy or C.V. could be detected.

### 3.6. Column performance

No precolumn has been used in this study. The number of plasma samples injected on the column during development and testing of the bioanalytical part exceeds 200. A fast deterioration of the column performance was observed when using 5 m*M* of DDEDMA in 0.01 *M* HCl for the dissolution of the residues before injection. Another effect of this solvent was an interfering system peak [21] on the chromatogram. The remedy for these problems was to remove the quaternary ammonium ion and dilute the concentration of HCl to 0.001 *M*. By washing the column with approximately 100 ml of 20% 2-propranol in Millipore water, the column performance was partially restored.

## 4. Conclusions

The proposed method for the simultaneous determination of (+)- and (−)-citalopram in plasma is rapid, reproducible, enantioselective and accurate. It

Table 4
Day-to-day reproducibility

| Day | Added (ng/ml) | Found (mean±S.D) (ng/ml) | C.V. (%) | Added (ng/ml) | Found (mean±S.D) (ng/ml) | C.V. (%) |
|---|---|---|---|---|---|---|
| *(−)-Citalopram* | | | | | | |
| Day 1 | (9.25) | 9.97±0.52 | 5.25 | (118) | 120±9.50 | 7.92 |
| Day 6 | (9.25) | 9.84±0.42 | 4.24 | (118) | 123±3.92 | 3.19 |
| Day 7 | (9.25) | 8.87±0.84 | 9.42 | (118) | 118±5.16 | 4.37 |
| Day 8 | (9.25) | 9.31±0.74 | 7.96 | (118) | 117±6.19 | 5.29 |
| Day 12 | (9.25) | 9.23±0.53 | 5.69 | (118) | 121±5.88 | 4.86 |
| Day 13 | (9.25) | 9.31±0.70 | 7.52 | (118) | 114±4.71 | 4.13 |
| *(+)-Citalopram* | | | | | | |
| Day 1 | (10.2) | 9.77±0.55 | 5.62 | (130) | 127±9.37 | 7.38 |
| Day 6 | (10.2) | 10.1±0.73 | 7.24 | (130) | 134±5.79 | 4.32 |
| Day 7 | (10.2) | 10.3±0.73 | 7.11 | (130) | 126±3.11 | 2.47 |
| Day 8 | (10.2) | 9.64±0.56 | 5.79 | (130) | 130±8.54 | 6.57 |
| Day 12 | (10.2) | 9.46±0.59 | 6.27 | (130) | 135±5.18 | 3.84 |
| Day 13 | (10.2) | 10.1±1.0 | 10.0 | (130) | 126±7.51 | 5.96 |

can be applied to pharmacokinetic studies of respective enantiomer as well as to therapeutic monitoring.

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