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# Stereoselective determination of pyridoglutethimide enantiomers in serum with a chiral cellulose-based high-performance liquid chromatographic column using solid phase extraction and UV detection

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**Abstract**

A sensitive method for the separation and determination of *R*(+)- and *S*(−) enantiomers of pyridoglutehimide in serum by high performance liquid chromatography (HPLC) with UV detection was developed. The assay involves the use of a solid-phase extraction for serum sample clean-up prior to HPLC analysis using a C18 Bond–Elute column. Chromatographic resolution of the enantiomers was performed on a reversed-phase cellulose-based chiral column (Chiralcel OD-R, 250 × 4.6 mm I.D.) under isocratic conditions using a mobile phase of 25:75 v/v acetonitrile-0.3 M aqueous sodium perchlorate (pH 6.2 adjusted with perchloric acid) at a flow rate of 0.8 ml/min. Recoveries for *R*(+)- and *S*(−)-pyridoglutethimide enantiomers were in the range 86–91% at 300–900 ng/ml level. Intra-day and inter-day precision calculated as %R.S.D. were in the ranges of 2.9–3.9 and 1.5–4.7% for both enantiomers, respectively. Intra-day and inter-day accuracies calculated as percentage error were in the ranges of 1.9–3.3 and 1.5–3.9% for both enantiomers, respectively. Linear calibration curves in the concentration ranges of 100–1500 ng/ml for each enantiomer show correlation coefficient (*r*) of more than 0.9995. The limit of quantification (LOQ) of each enantiomer was 100 ng/ml using 1 ml of serum. The detection limit (LOD) for each enantiomer in serum using a UV detection set at 257 nm was 50 ng/ml (*S*/*N* = 2).


*Keywords:* Pyridoglutethimide enantiomers; Chiralcel OD-R; Solid phase extraction; Serum

## 1. Introduction

The utmost importance of the stereochemistry of compounds with regard to their interaction with biological targets is generally recognized. Stereoisomers often differ substantially in their pharmacological, toxicological or pharmacoki-

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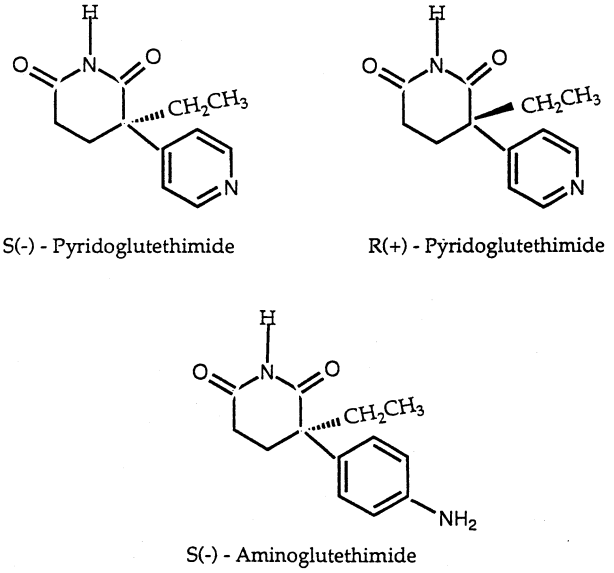


Fig. 1. Chemical structure of pyridoglutethimide enantiomers and $S(-)$-aminoglutethimide (I.S.).

netic profiles. This fact has also influenced the requirements of the regulatory authorities towards chiral compounds [1–3]. As a consequence, the determination of the stereochemical purity and/or the stereochemical composition of a compound is an important issue for the pharmaceutical, chemical and cosmetic industry as well as the biological and environmental analyses [3].

The antitumor effects of pyridoglutethimide, 3-ethyl-3(4-pyridyl) piperidine-2,6-dione, a new analogue of aminoglutethimide, were examine by Perez and Borja [4] and Yamamoto et al. [5], first synthesized by Foster et al. [6]. Aminoglutethimide is an agent, which is clinically used for the treatment of hormone-dependent tumors acting through inhibition of the aromatase enzyme [7,8]. However, aminoglutethimide produces several neurological side-effects e.g. sedation, ataxia among others, and also interferes with general steroid biosynthesis through inhibition of the desmolase enzyme system (inhibition of side-chain cleavage in cholesterol). Hence, hydrocortisone must be administered as a replacement therapy [9]. Pyridoglutethimide exhibits a strong competitive inhibitory activity, which is selective against the aromatase enzyme system while it does not inhibit the desmolase enzyme system, therefore, there is no need for hydrocortisone administration. Furthermore, pyridoglutethimide produces little or no neurotoxic side effects [10]. It is currently in clinical trials in postmenopausal women with hormone dependent metastatic breast carcinoma and is considered a more effective and selective chemotherapeutic agent than aminoglutethimide.

Pyridoglutethimide has an asymmetric center at the three position (Fig. 1) and is clinically administered as the racemic mixture, although the $R(+)$ enantiomer is more active than $S(-)$ enantiomer [11]. Chemical resolution of racemic pyridoglutethimide was previously reported using a chiral stationary phase based on the $(R,R)$ tartaramide [12]. Aboul-Enein et al. [13] have described a separation of $R,S$-pyridoglutethimide using either cellulose-based chiral stationary phases (Chiralcel OD and Chiralcel OJ) operated in the normal phase mode or Chiralcel OJ-R column operated in the reversed phase mode [14]. Recently, pyridoglytethimide was determined in serum utilizing either Chiralcel OJ-R column [15] or capillary electrophoresis using alpha-cyclodextrine as chiral selector [16]. Cellulose tris-(3,5-dimethyl phenyl carbamate) known as Chiralcel OD-R has proved efficient in the direct resolution and determination of several drugs enantiomers in serum [17–30]. Chiral selectivity in OD-R column involve similar interactions as their normal phase counterparts such as hydrogen bonding, dipole–dipole interactions, Π–Π interaction and the formation of inclusive complexes. However, the OD-R column is more suitable than the OD column for the analysis of chiral drugs in serum since many chiral drugs exist as salt forms and are more water soluble. No method has yet been reported to determined the levels of the pyridoglutethimide enantiomers in serum using OD-R column.

In this paper, a sensitive and stereospecific assay for the quantification of pyridoglutethimide enantiomers in serum is described using the cellulose-based chiral stationary phase Chiralcel OD-R and solid-phase extraction. The method is linear over the range 100–1500 ng/ml with UV detector at 257

nm. The detection limit (LOD) for each enantiomers was 50 ng/ml ($S/N = 2$).

## 2. Experimental

### 2.1. Reagents and chemicals

Powdered samples of $R(+)$- and $S(-)$-pyridoglutethimide and the internal standard $S(-)$ aminoglutethimide were kindly supplied by Dr M. Stogniew (U.S. Bioscience Inc., West Conshohocken, PA, USA). Blank bovine serum (Cat #3160-34) was purchased from Instrumentation Lab (Lexington, MA, USA). High performance liquid chromatography (HPLC) grade acetonitrile and 60–62% perchloric acid were obtained from J.T. Baker (Philipsburg, NJ, USA). HPLC-grade sodium perchlorate was obtained from Fisher Scientific (Pittsburgh, PA, USA) C18, C8, cyanopropyl and phenyl solid-phase extraction columns (100 mg per 1 $cm^3$ size) were obtained from Varian Sample Preparation Products (Harbor City, CA, USA). The Vac-Elut vacuum manifold was obtained from analytichem international (Sunnyvale, CA, USA). All chromatographic solution were filtered through a 0.45 um filter (Alltech, Deerfield, IL, USA) and degassed by sonication prior to use.

### 2.2. Chromatographic conditions

Chromatography was performed on an isocratic HPLC system consisting of a Beckman Model 110A solvent delivery module (Beckman, SanRamon, CA, USA) equipped with a 100 ul loop, and spectroflow 757 absorbance detector (Kratos Analytical Ramesy, NJ, USA) set at 257 nm. Data acquisition was performed on a HP Model 3290 integrator (Hewlett–Packard, Avondale, PA, USA).

Separations were accomplished on a reversed phase cellulose-based chiral column (Chiralcel OD-R, 10 um, 250 mm × 4.6 mm I.D.) obtained from Chiral Technologies, (Exton, PA, USA) and operated at ambient temperature ($23 \pm 1$ °C). The mobile phase consisted of 25:75 v/v acetonitrile-0.3 M aqueous sodium perchlorate (pH 6.2 adjusted with 60–62% perchloric acid) and was delivered at a flow rate of 0.8 ml/min.

### 2.3. Preparation of stock and spiked standard solutions

Individual stock solutions of $R(+)$- and $S(-)$-pyridoglutethimide and the internal standard $S(-)$-aminglutethimide were prepared in deionized water to give concentration of 100 ug/ml and storage protected from light at 4 °C. Under these conditions, stock solution of pyridoglutethimide enantiomers and internal standard were stable for at least 2 weeks. Appropriate volume of the $R(+)$- and $S(-)$-pyridoglutethimide and the internal standard stock solutions were pipetted into a 1 ml volumetric tubes and serum was added to volume to give final concentration of 100, 200, 400, 800, 1200 and 1500 ng/ml of each analyte and 5000 ng/ml of the internal standard.

### 2.4. Assay method

Bond-Elute C18 solid phase extraction (SPE) cartridges were attached to a vacuum manifold and conditioned with two column volumes of absolute methanol followed by two column volumes of distilled water (note: do not allow sorbent to dry). Into the cartridges were transferred blank and spiked serum samples and the vacuum was applied. After the entire serum sample had been aspirated through the cartridge, the cartridge was washed with 4 × 500 ul of distilled water and then dried under full vacuum for 5 min. The pyridoglutethimide enantiomers and internal standard were eluted with 4 × 250 ul of absolute methanol. The eluent was evaporated to dryness under a nitrogen stream at ambient temperature. The residue was reconstituted in 1 ml of mobile phase and triplicate 100 ul injections were made into the liquid chromatographic system. Linear regression analysis of enantiomer concentration versus peak-area ratio of each pyridoglutethimide enantiomer to internal standard produced slope and intercept data which were used to calculate concentration of $R(+)$-and $S(+)$-pyridoglutethimide enantiomers in each serum sample.

## 3. Results and discussion

Reversed-phase cellulose-based chiral stationary phases employ the same chiral selectors as their normal phase counterparts (e.g. Chiralcel OD-R versus Chiralcel OD), but are designed for analytical applications using reversed-phase chromatography. The normal-phase mode, however, has some disadvantages over the reversed-phase mode. First, normal-phase HPLC utilizes a nonpolar mobile phase such as hexane, chloroform and methylene chloride, which are highly inflammable and environmentally harmful solvents. Second, extracts of biological fluids including serum and urine contain many endogenous polar substances, which are strongly retained on normal-phase columns and would shorten the life time of the column. Thus, complex and time consuming clean-up procedures would be required to remove these interfering substances [31,32].

### 3.1. Optimization of the chromatographic conditions

In reversed-phase HPLC, polar substances are weakly retained on the column and are eluted rapidly. Furthermore, aqueous buffers used as component of a mobile phase will permit direct introduction of aqueous samples onto the column as well as regulation of the retention by changing the mobile phase composition (pH, ionic strength, or content of organic modifier).

A successful resolution of the pyridoglutethimide enantiomers was achieved in the reversed-phase mode on the Chiralcel OD-R column. Initial resolution of the enantiomers ($Rs = 1.0$) with retention times of 20–25 min was obtained using a mobile phase consisting of 0.5 M aqueous sodium perchlorate–acetonitrile (60:40 v/v). The influences of buffer type, pH, concentration and amounts of acetonitrile in the mobil phase on resolution of the analytes were investigated. The function of buffer in a column of this type is to suppress ionization of acidic and basic analytes since ionization of analytes causes deformation and tailing of the peaks. An increase in the concentration of acetonitrile in the mobile phase reduced retention times with some loss of resolution of the analytes of interest.

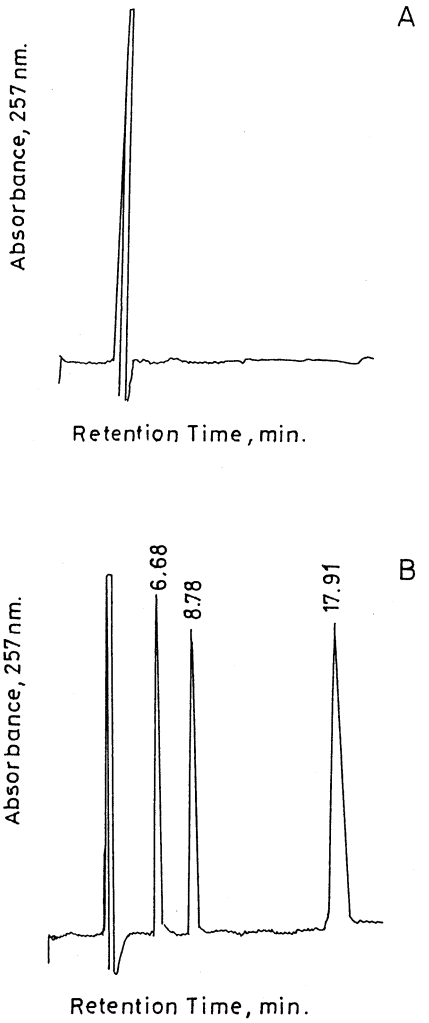


Fig. 2. Typical chromatograms of (A) blank serum and (B) serum spiked with $R(+)$-pyridoglutethimide (6.88 min), $S(-)$-pyridoglutethimide (8.76 min) and internal standard (17.91 min) on a Chiralcel OD-R column using a 25:75 v/v acetonitrile–aqueous sodium perchlorate pH 6.2 mobile phase with detection at 257 nm.

Whether a buffer or deionized water was used in the mobile phase was dependent on the internal standard used. If the internal standard was an acidic or basic compound, a mobile phase consisting of a buffer–acetonitrile instead of deionized water–acetonitrile was employed to prevent the internal standard peak from being deformed. The

Table 1
Chromatographic parameter data for pyridoglutethimide enantiomers and internal standard in spiked serum

| Analyte | Rs | $\alpha$ [a] | (mean ± S.D., $n = 6$) | | |
|---|---|---|---|---|---|
| | | | $K^-$ | $T_R$ (min) | $N$ [b] |
| $R(+)$-pyridoglutethimide | 3.94 | 1.59 | 0.91 ± 0.03 | 6.77 ± 0.16 | 2443 ± 36 |
| $S(-)$-pyridoglutethimide | 9.66 | 2.69 | 1.45 ± 0.06 | 8.77 ± 0.04 | 1794 ± 49 |
| $S(-)$ aminoglutethimide | | | 3.91 ± 0.05 | 17.90 ± 0.08 | [c] |

[a] Separation factor, calculated as $K_2^-/K_1^-$.
[b] Theoretical plates, calculated as $N = 16\ (T_R/w)^2$.
[c] Not calculated.

final mobile phase selected for this separation consisting of 0.3 M sodium perchlorate–acetonitrile (75:25 v/v) and provided good peak shape for the baseline separation of the two enantiomer peaks (Rs = 3.9) with suitable retention times (7–9 min for pyridoglutethimide enantiomers and 18 min for the internal standard), and sensitivity in the desired ng/ml range.

Typical HPLC chromatograms for blank serum and serum spiked with 1200 ng/ml of each enantiomer and 5000 ng/ml of internal standard are shown is Fig. 2. No interference were observed in blank serum at the retention times of $R(+)$- and $S(-)$-pyridoglutethimide peaks. The selection of an internal standard was difficult, some neutral compounds with an amine group in their structure were tested, but $S(-)$ aminoglutethimide was finally selected as internal standard based on good recovery from serum (84%), a suitable retention time (18 min.) and a separation factor ($\alpha$) of 2.7 between $S(-)$-aminoglutethimide and the last eluting $S(-)$-pyridoglutethimide enantiomer. Quantification was based on the plot of concentration of each enantiomer versus peak-area ratios of each pyridoglutethimide enantiomers to the internal standard.

The suitability of the Chiralcel OD-R for the separation of the pyridoglutethimide enantiomers is shown in Table 1. The retention times of $R(+)$- and $S(-)$-pyridoglutethimide and internal standard $S(-)$-aminoglutethimide were 6.77 ± 0.16, 8.77 ± 0.04 and 17.90 ± 0.08 min, respectively ($n = 9$). Relative retention of the $R(+)$-and $S(-)$-pyridoglutethimide enantiomers was expressed by the separation factor $\alpha$ of 1.6. Resolution (Rs) of the $R(-)$- and $S(-)$-pyridoglutethimide enantiomers peaks and the last eluting $S(-)$ enantiomer and the internal standard peaks were 3.9 and 9.6, respectively.

Table 2
Analytical parameters for the determination of pyridoglutethimide enantiomers using the proposed method

| Parameter | $R(+)$-pyridoglutethimide | $S(-)$-pyridoglutethimide |
|---|---|---|
| Concentration range (ng/ml) | 100–1500 | 100–1500 |
| Regression equation | | |
| Intercept ($a$) | 0.0483 | 0.0167 |
| Slope ($b$) | 0.0048 | 0.0046 |
| Correlation coefficient ($r$) | 0.9995 | 0.9998 |
| $S_{y/x}$ | 0.5079 | 0.4862 |
| $S_a$ | 0.6525 | 0.6139 |
| $S_b$ | 0.3216 | 0.3099 |
| LODs (ng/ml) | 50.0 | 50.0 |

### 3.2. Application to spiked serum

In the course of developing a SPE procedure for serum sample clean up, three solid-phase extraction cartridges (C18, C8 and cyanopropyl) were investigated. The cyanopropyl cartridge showed interfering endogenous serum peaks at 6.5and 8.5 min which co-eluted with $R(+)$- and $S(-)$-pyridoglutethimide peaks. An octyl (C8) SPE column was also found to be unacceptable due to co-elution of endogenous serum components with

Table 3
Accuracy and precision data for pyridoglutethimide enantiomers in serum

| Analyte | Conc. add (ng/ml) | Conc. found (ng/ml) | Error (%) | R.S.D. (%) |
|---|---|---|---|---|
| | 300 | 305.79 ± 8.77 | 1.9 | 2.9 |
| *Intra-day* | 600 | 579.99 ± 18.84 | 3.3 | 3.2 |
| *R*(+)-pyridoglutethimide | 900 | 920.70 ± 32.96 | 2.3 | 3.6 |
| | 300 | 306.39 ± 9.97 | 2.1 | 3.3 |
| *S*(−)-pyridoglutethimide | 600 | 586.98 ± 22.95 | 2.2 | 3.9 |
| | 900 | 877.50 ± 29.61 | 25 | 3.4 |
| *Inter-day* | 300 | 304.49 ± 4.61 | 1.5 | 1.5 |
| *R*(+)-pyridoglutethimide | 600 | 576.69 ± 27.31 | 3.9 | 4.7 |
| | 900 | 887.86 ± 31.64 | 1.3 | 3.6 |
| | 300 | 305.47 ± 6.73 | 1.8 | 2.2 |
| *S*(−)-pyridoglutethimide | 600 | 579.48 ± 19.20 | 3.4 | 3.3 |
| | 900 | 915.45 ± 24.50 | 1.7 | 2.7 |

Mean ± S.D. based on $n$, 3; Mean ± S.D. based on $n$, 9.

*R*(+)-pyridoglutethimide enaniomers peak at 6.2 min. An octadecyl (C18) SPE column provided the best results in terms of clean-up and recoveries of *R*(+)- and *S*(−)-pyridoglutethimide enantiomers. Fig. 2A and B shows the chromatogram of blank serum and spiked serum containing *R*(+)-and *S*(−)-pyridoglutethimide.

### 3.3. Validation

The method was tested for linearity, specificity, precision and reproducibility. By using the above chromatographic procedure, linear regression equations were obtained. The regression plots showed that there was a linear dependence of the peak-area on the concentration of both enantiomers over the ranges given in Table 2. The table also shows the LOD and the results of the statistical analysis of the experimental data, such as the slopes, the intercepts, the correlation coefficients obtained by the linear least squares treatment of the results along with standard deviation of the slope ($S_b$) and intercept ($S_a$) on the ordinate and the standard deviation of the residuals ($S_{y/x}$). The good linearity of the calibration graphs and the negligible scatter of the experimental points are clearly evident by the values of the correlation coefficients and standard deviations [33].

The specificity of the method was investigated by observing any interference encountered from the endogenous serum components. It was shown that these components do not interfere with the proposed method (Fig. 2). To examine the ruggedness of the procedure, the precision and accuracy (percent error) of the method were determined by using serum samples spiked at 300, 600, 900 ng/ml levels (Table 3). The data indicate that intra-day precision was in the 2.9–3.9% range ($n = 3$) and intra-day accuracy in the 1.9–3.3% range ($n = 3$) for both pyridoglutethimide enantiomers and that inter-day precision was in the 1.5–4.7% range ($n = 9$) and inter-day accuracy in the 1.5–3.9% range ($n = 9$) for both pyridoglutethimide enantiomers.

The minimum detectable concentration (LOD) of each enantiomer of pyridoglutethimide was determined to be 50 ng/ml ($S/N = 2$). The lowest quantifiable level (LOQ) was found to be 100 ng/ml for each enantiomer. The robustness of the method is demonstrated by the versatility of the experimental factors that affect the peak-area.

## 4. Conclusion

In summary, an HPLC method has been developed and validated for the assay of *R*(+)- and *S*(−)-pyridoglutethimide enantiomers in serum using a Chiralcel OD-R column operated in the reversed-phase mode. The method utilized a C18 cartridge SPE for sample clean-up of serum. The procedure is suitable for the separation and

quantification of each enantiomer of pyridoglutethimide in 100–1500 ng/ml range.

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