Determination of Aminoglutethimide Enantiomers as Dansyl Derivative in Human Plasma by HPLC with Fluorescence Detection [1)]

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Summary

Determination of aminoglutethimide enantiomers as a dansyl derivative in plasma by HPLC has been achieved using cellulose tris(3,5-dimethylphenyl carbamate) chiral stationary phase known as Chiralcel OD, and a mobile phase consisted of ethanol-cyclohexane-methanol (95:5:2 *v/v/v*). The limit of detection for each enantiomer of aminoglutethimide using fluorescence detector was 20 ng ml^{-1}.

Introduction

Aminoglutethimide (±AG), ±-3-(4-aminophenyl)-3-ethyl-2,6-piperidinedione, was initially developed as an anticonvulsant for the treatment of epilepsy, but was subsequently withdrawn because of its inhibitory effects on adrenal function. *Rac*-aminoglutethimide is currently used clinically as a drug of choice in the treatment of hormone-dependent metastatic breast cancer. It was reported that the (+)-*R*-isomer had the greatest steroidogenesis inhibitory activity (two or three times more potent than the racemate), while the (–) S-isomer had very little activity at dose levels 10-fold higher [1].

Direct resolution of *rac*-aminoglutethimide and its acetylated metabolite has been reported by HPLC on various chiral stationary phases such as Chiralcel OD and OJ columns [2,3], a tris(4-methylbenzoate)cellulose covalently bonded to an aminosilica support [4], α_1-acid glycoprotein (α_1-AGP) column [5], and recently on vancomycin chiral stationary phase [6].

Several non-chiral [7,8] and chiral assays [9] have been described for analysis of *rac*-aminoglutethimide and its enantiomers in plasma.

Dansyl derivatization has been widely employed for the analysis of aminoacids [10] and amines [11,12]. These dansyl derivatives can be separated by HPLC and detected sensitively by UV or fluorescent detectors. Enantiomers of dansyl amino acids have been separated using β-cyclodextrin on an ODS column [13]. Polysaccharides have also proved to be valuable chiral selectors for derivatives of racemic compounds [14].

Derivatization of the amino group of *rac*-AG with dansyl chloride provides the molecule with additional interaction sites and thus improves detectability. This paper describes the enantioselective separation and determination of aminoglutethimide enantiomers after derivatization with dansyl chloride in plasma using on a cellulose tris (3,5-dimethylphenyl carbamate) chiral stationary phase known as Chiralcel-OD and fluorescence detector. The effect of solvents on the resolution of dansyl derivative of aminoglutethimide enantiomers was studied.

Results and Discussion

Derivatization of AG and internal standard proved to be optimal and reproducible under the conditions described above. In order to improve the resolution of the enantiomers, several mobile phase compositions were tested (Table 1). The best results in terms of resolution, analysis time, and separation factor were obtained with mobile phase consisted of ethanol-cyclohexane-methanol (95:5:2 *v/v/v*). The use of cyclohexane led to an improvement in stereoselectivity [15]. The sensitivity of fluorescence detector was nearly 7 fold higher than that of UV detector.

Typical retention times (standard deviations, S.D.) for IS, (*R*)AG-DNS, and (*S*)AG-DNS were 7.250 (±0.007), 8.970 (±0.024), and 11.560 (±0.059), respectively ($n = 20$). Inter-day differences in mobile phase composition did not significantly alter chromatographic performance.

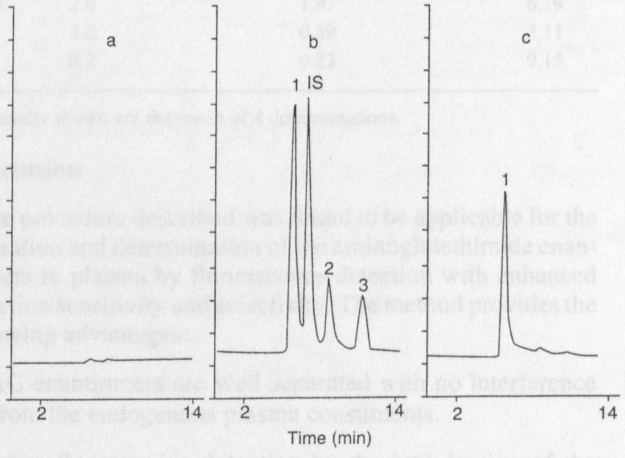

Figure 1. Representative chromatograms of human plasma: a) blank human plasma, b) human plasma spiked with *rac*-AG at 4 µg ml^{-1} and IS; c) human plasma spiked with dansyl chloride and sodium bicarbonate. 1, Dansyl chloride; IS, Internal standard; 2, (+)-*R*-dansylAG; 3 , (–)-*S*-dansylAG

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Table 1. Enantiomeric separation of racemic dansyl aminoglutethimide on Chiralcel OD column.

	Mobile phase composition	Chromatographic parameters[a]			
		k_1'	k_2'	α	R_s
I	Ethanol-*n*-hexane (95:5 *v/v*)	1.23	1.78	1.45	3.86
II	Ethanol-cyclohexane (95:5 *v/v*)	1.20	1.74	1.45	4.00
III	Ethanol-cyclohexane-methanol (95:5:2 *v/v/v*)	1.22	1.86	1.52	4.42
IV	Ethanol-cyclohexane-methanol (95:5:3 *v/v/v*)	1.19	1.78	1.49	4.35
V	Ethanol-isopropanol-cyclohexane (70:10:20 *v/v/v*)	1.24	1.64	1.32	2.56
VI	Ethanol-cyclohexane (90:10 *v/v*)	1.18	1.57	1.33	2.90

[a] k_1', k_2': Capacity factor of the first and second eluting enantiomer; α: Separation factor; R_s: Resolution factor ($R_s = 1.18\ (t_2 - t_1)/w_1 + w_2$).

Chromatograms representing a blank plasma sample and plasma spiked with 4 μg ml^{-1} *rac*-AG and internal standard are presented in Figure 1. There was no interference from endogenous plasma constituents.

Table 2. Linearity of the assay.

Enantiomer	Correlation coefficient	Slope	Intercept
R-AG	0.999	0.1298	0.027
S-AG	0.999	0.0895	0.089

The results are the mean of 6 determinations.

Linearity

The calibration curves for *R*-AG, and *S*-AG in plasma were linear in the concentration ranges 0.3–3.0 μg ml^{-1} for AG. Plasma concentrations were derived from linear regression analysis of the peak height ratios (analyte/IS) vs. concentration curves (Table 2).

Precision and Accuracy

The recovery of each enantiomer from plasma was determined using the ratio of peak area observed for the enantiomer extracted from plasma to that obtained from the direct injection of the same amount of the enantiomer in dichloromethane. The inter-assay variation was determined over a range of concentrations shown in Table 3. The results were similar to the intra-assay variation shown in Table 4.

The coefficient of variations (C.V.) for intra and inter-day precision were in the ranges 1.77–10.42% (Tables 3 and 4). The lower limit of detection (signal to noise ratio of 3:1) was found to be 20 ng ml^{-1} for each AG enantiomer using a 300 μl plasma sample size.

Table 3. Inter-assay precision and accuracy for the analysis of AG enantiomers as dansyl derivatives in plasma.

Concentration added (ng ml^{-1})	*R*-Aminoglutethimide		*S*-Aminoglutethimide	
	Mean (%)	C.V. (%)	Mean (%)	C.V. (%)
860	87.1	1.87	86.5	1.77
430	84.2	3.47	83.6	2.61
215	79.6	4.51	80.4	5.60

The results are the mean of 6 determinations.

Table 4. Intra-day precision and accuracy for the analysis of AG enantiomers as dansyl derivatives in plasma

Enantiomer	Concentration added (μg ml^{-1})	Experimental concentration found (μg ml^{-1})	C.V. (%)
R-AG	2.0	1.89	6.18
	1.0	0.91	4.48
	0.2	0.23	10.42
S-AG	2.0	1.97	6.19
	1.0	0.89	3.11
	0.2	0.22	9.15

The results shown are the mean of 4 determinations.

Conclusion

The procedure described was found to be applicable for the separation and determination of the aminoglutethimide enantiomers in plasma by fluorescence detection with enhanced detection sensitivity and selectivity. The method provides the following advantages:

a) AG enantiomers are well separated with no interference from the endogenous plasma constituents.

b) using fluorescence detection by the introduction of the dansyl group improves the response at least 7 folds that of UV absorbance, and also eliminates interference which could be detected by UV absorbance.

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Experimental

Materials

Rac-aminoglutethimide (±AG) was kindly supplied by Ciba-Geigy (Basle, Switzerland). Dansyl chloride (DNS-Cl) was purchased from Aldrich (Milwaukee, Wisconsin, USA). 2-(3,4-dimethoxyphenyl)ethylamine, HPLC grade isopropanol, methanol dichloromethane and *n*-hexane were obtained from E. Merck (Darmstadt, Germany) and cyclohexane was obtained from Fischer Scientific (Fairlawn, NJ, U.S.A.).

Instrumentation and Assay Conditions

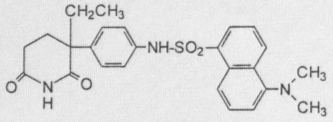
Chromatography was performed using a liquid chromatographic system consisting of a Waters Model 420 Fluorescence detector ($\lambda_{excitation}$: 360 nm, $\lambda_{emission}$: 530 nm) and Model 440 UV 254 nm detector, a U6K injector, a 510 Model pump, a Hewlett-Packard 3365 ChemStation data analysis system, and a Chiralcel OD column (25 cm × 4.6 mm i.d., coated on a silica gel, 10 µm particle size) was purchased from Daicel (Tokyo, Japan).

The elution order and peak identification for dansyl AG enantiomers was performed with Shodex OR-1 optical rotation detector (JM Science Inc., Buffalo, NY, USA) using the same chromatographic set up described above.

Derivatization of Aminoglutethimide (AG-DNS)

10^{-3} M of AG in 10ml of acetone was refluxed with 10^{-3} M of DNS-Cl in the presence of 77.5 mg sodium bicarbonate for 30 min at 45 °C. The precipitate, 1-4-(3-ethyl-2,6-dioxo-3-piperidyl)phenyl-5-dimethylaminonaphthalenesulfonamide, was filtered and crystallized from ethyl acetate (yield 66.6%). Yellow crystals. m.p. 203 °C. The derivative was identified by IR, ^{1}H-NMR spectrometry, and elemental analysis.

^{1}H-NMR (DMSO): δ = 0.74 (t, 3H, CH_2CH_3); 1.81 (m, 2H, CH_2-CH_3); 2.12 (m, 4H, CH_2-CH_2); 2.98 (6H, $N(CH_3)_2$); 7.21 (d, J = 8.6 Hz, 2H, aromatic H); 7.28 (d, J = 8.8 Hz, 2H, aromatic H); 7.42 (d, 1H, naphthalene H); 7.77 (dd, 2H, naphthalene H); 8.41 (d, 1H, naphthalene H); 8.51 (d, 1H, naphthalene H); 8.63 (d, 1H, naphthalene H); 10.86 (s, 1H, NH); 10.96 (s, 1H, NH).– Anal.: $C_{25}H_{27}N_3O_4S$.



Sample Preparation

Stock solutions of *rac*-AG (40 µg ml^{-1}) and 2-(3,4-dimethoxyphenyl)-ethylamine (IS) (0.272 µg ml^{-1}) were freshly prepared in dichloromethane. Working solutions were obtained by diluting stock solutions in dichloromethane. Plasma controls were prepared by spiking blank plasma with known amounts of *rac*-AG.

Plasma Analysis

Plasma calibration standards were prepared fresh daily by spiking 300 µl of blank human plasma with (10–100 µl) of *rac*-AG standard solutions and 100 µl of IS solution of 2-(3,4-dimethoxyphenyl)ethylamine. Acetonitrile (100 µl) was added for protein precipitation. The samples were buffered to pH 5.6 with 250 µl of 0.1 M acetate buffer.

The analytes were extracted from plasma by adding 4 ml of dichloromethane and vortex-mixing for 1 minute. The samples were centrifuged for 10 min at 1900 *g* to separate the phases. The upper layer was aspirated, and 2.5 ml of the lower organic phase was transferred to a conical glass tube. DNS-Cl solution 250 µl (0.4 mg ml^{-1} in acetone) and 20 mg of sodium carbonate were added, vortexed for 1 min and kept at room temperature in the dark for one hour to complete dansylation. An aliquot (500 µl) was evaporated under nitrogen, reconstituted in 250 µl of mobile phase and vortexed for 1 min, 20 µl of the sample was injected into the column. The fluorescence obtained was stable for at least 12 h.

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