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Short Communication

The determination of (*R*)- and (*S*)-glutethimide and the corresponding 4-hydroxyglutethimide metabolites in human serum and urine using a Pirkle-type HPLC chiral stationary phase

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Introduction

Glutethimide (Fig. 1) is a sedative–hypnotic drug, which is not widely prescribed because of the severe side effects associated with its use. One of these side effects is acute intoxication caused by overdoses of glutethimide. In some cases of glutethimide poisoning, the plasma half-life of the drug may exceed 100 h and the patients often show cyclic variations in the level of intoxication [1].

The metabolism of glutethimide has been shown to play an important role in the incidence and severity of the toxicity of the drug [2–4]. One of the metabolites, 4-hydroxyglutethimide, possesses marked sedative hypnotic activity and has been shown to accumulate in the plasma of drug-intoxicated humans [2–4]. In fact, work by Kennedy *et al.* [4] on the metabolism of glutethimide in intoxicated patients has demonstrated that the plasma levels of unconjugated 4-hydroxyglutethimide were significantly higher than those of any of the other metabolites.

Glutethimide is a chiral molecule which is administered as a racemic mixture. Initial work on the metabolism of this drug suggested that the metabolism is stereospecific with

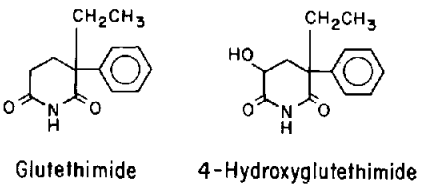

Figure 1
The structures of glutethimide and 4-hydroxyglutethimide.

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the (*R*)-(+)-enantiomer undergoing ring hydroxylation to the 4-hydroxy metabolite and the (*S*)-(−)-enantiomer undergoing side-chain hydroxylation [5]. Kennedy and Fischer [6] reinvestigated the stereospecificity of glutethimide metabolism in humans by administering the drug as either the racemic mixture or as the separate enantiomers. They did not observe a stereochemical preference in the formation of 4-hydroxyglutethimide.

Whilst the stereospecificity of glutethimide metabolism has been studied in healthy subjects, it has not been investigated in intoxicated patients. This is due to the lack of an analytical method capable of measuring (*R*)-(+)- and (*S*)-(−)-glutethimide and the corresponding 4-hydroxy metabolites in the same sample. A solution to this problem was suggested by Yang *et al.* [7] who reported the resolution of (*R*)-(+)- and (*S*)-(−)-glutethimide on a HPLC chiral stationary phase (HPLC–CSP) composed of (*S*)-*N*-(3,5-dinitrobenzoyl)leucine bound to an aminopropyl silica.

The present communication describes the development of an analytical method which is capable of measuring the concentrations of (*R*)-(+)- and (*S*)-(−)-glutethimide and the corresponding 4-hydroxy metabolites in the same sample. The method utilizes a commercially available form of the (*S*)-*N*-(3,5-dinitrobenzoyl)leucine HPLC–CSP and a hexane–2-propanol–acetonitrile gradient and has been used to analyze both plasma and urine samples from glutethimide intoxicated patients.

Experimental

Apparatus

A Spectra-Physics (San Jose, CS, USA) liquid chromatograph consisting of a model 8700 solvent delivery system, a Model 8773 Variable Wavelength Detector set at 220 nm and a Model 4200 Computing Integrator was used. A stainless steel column (25 cm × 4.6 mm i.d.) packed with (*S*)-*N*-(3,5-dinitrobenzoyl)leucine covalently bound to 5 μm aminopropyl silica (J. H. Baker, Phillipsburg, NJ, USA), thermostatted a Lazar HPLC water jacket (Bodman Chemicals, Media, PA, USA) and a Forma Model 20006 circulating water bath (VWR Scientific, San Francisco, CA, USA) was used.

Materials

The glutethimide reference standard was supplied by the United States Pharmacopoea (Rockville, MD, USA) and the 4-hydroxyglutethimide was synthesized according to the method of Aboul-Enein *et al.* [8] and kindly supplied by L. Fischer. The oxazolidone of methoxamine was synthesized using a previously described method [9]. HPLC grade hexane, 2-propanol and acetonitrile were purchased from J. T. Baker. All other chemicals were reagent grade and used as purchased.

Serum and urine samples

The serum and urine samples analyzed in this study were provided by L. Fischer from samples collected in previous studies [2–4, 6]. The samples had been stored at −20°C and were defrosted just before analysis.

General procedure — serum

A serum sample (0.5 ml) was combined with 1 ml phosphate buffer (0.5 M, pH = 7.4) and 10 μl internal standard solution (oxazolidone of methoxamine, 100 μg/ml in 2-propanol). The solution was vortexed with 3 ml anhydrous ether and centrifuged at

2000 rpm for 5 min. The mixture was then placed in a dry ice–acetone bath and the unfrozen supernatant decanted and evaporated to dryness under nitrogen. The resulting solid was redissolved in 100 μl 2-propanol and 50 μl of the resulting solution were injected onto the HPLC column.

General procedure — urine

An aliquot (2 ml) of a urine sample was combined with 1 ml phosphate buffer (0.5 M, pH = 7.4) and 10 μl internal standard solution (oxazolidone of methoxamine, 100 μg/ml in 2-propanol). The solution was vortexed with 5 ml anhydrous ether and centrifuged at 2000 rpm for 5 min. The mixture was then placed in a dry ice–acetone bath and the unfrozen supernatant decanted and evaporated to dryness under nitrogen. The resulting solid was redissolved in water (2 ml) and chromatographed using a 1-ml C18 solid phase extraction column (J. T. Baker). Before the column was charged with the sample, it was conditioned with 2 ml of methanol followed by 2 ml carbonate buffer (0.1 M, pH = 10.5). The sample was then added to the column and the column washed with 2 ml of water and the desired compounds eluted with 2 ml methanol. The methanol was evaporated under nitrogen, the resulting solid redissolved in 110 μl 2-propanol and 50 μl of the resulting solution were injected onto the HPLC column.

Chromatographic conditions

Separations were achieved using a (*S*)-*N*-(3,5-dinitrobenzoyl)-leucine modified silica column (25 cm × 4.6 mm i.d.), particle diameter 5 μm with hexane–2-propanol–acetonitrile gradient elution using the profile: 0–30 min (96.5:2.5:1.0, v/v/v); 30–35 min (96.5:2.5:1.0 to 96:6:2, v/v/v); 35–100 min (92:6:2, v/v/v). A flow rate of 1 ml/min and a column temperature of 20°C were maintained throughout the analysis.

Standard curves

Standard curves for (*R*)- and (*S*)-glutethimide and the enantiomers of 4-hydroxy-glutethimide were constructed by adding known amounts of the compounds to water, serum and urine. For the serum standard curves, the concentrations of each of the four isomers ranged from 1 to 20 μg/ml. The plots of integrator count versus concentration for each compound were found to be linear over the concentration range examined. The (*R*)-glutethimide calibration had a standard error of 0.0466 and a correlation coefficient of 0.9968; the (*S*)-glutethimide curve values of 0.0411 and 0.9969; the first eluting 4-hydroxyglutethimide peak 0.0162 and 0.9985; and the second eluting 4-hydroxy-glutethimide peak 0.039 and 0.9957. For the urine standard curve, the concentrations of the glutethimide isomers ranged from 10 to 50 μg/ml and the concentrations of the 4-hydroxyglutethimide isomers ranged from 20 to 100 μg/ml. The calibration plots for each compound were linear over the concentration range. The (*R*)-glutethimide standard curve had a standard error of 0.0292 and a correlation coefficient of 0.9980; the (*S*)-glutethimide curve values of 0.0509 and 0.9950; the first eluting 4-hydroxyglutethimide peak 0.0170 and 0.9982; and the second eluting 4-hydroxyglutethimide peak 0.0475 and 0.9954.

Results and Discussion

A chromatogram of a plasma sample spiked with racemic glutethimide (10 μg/ml) and racemic 4-hydroxyglutethimide (12.5 .147g/ml) is presented in Fig. 2B. Although the

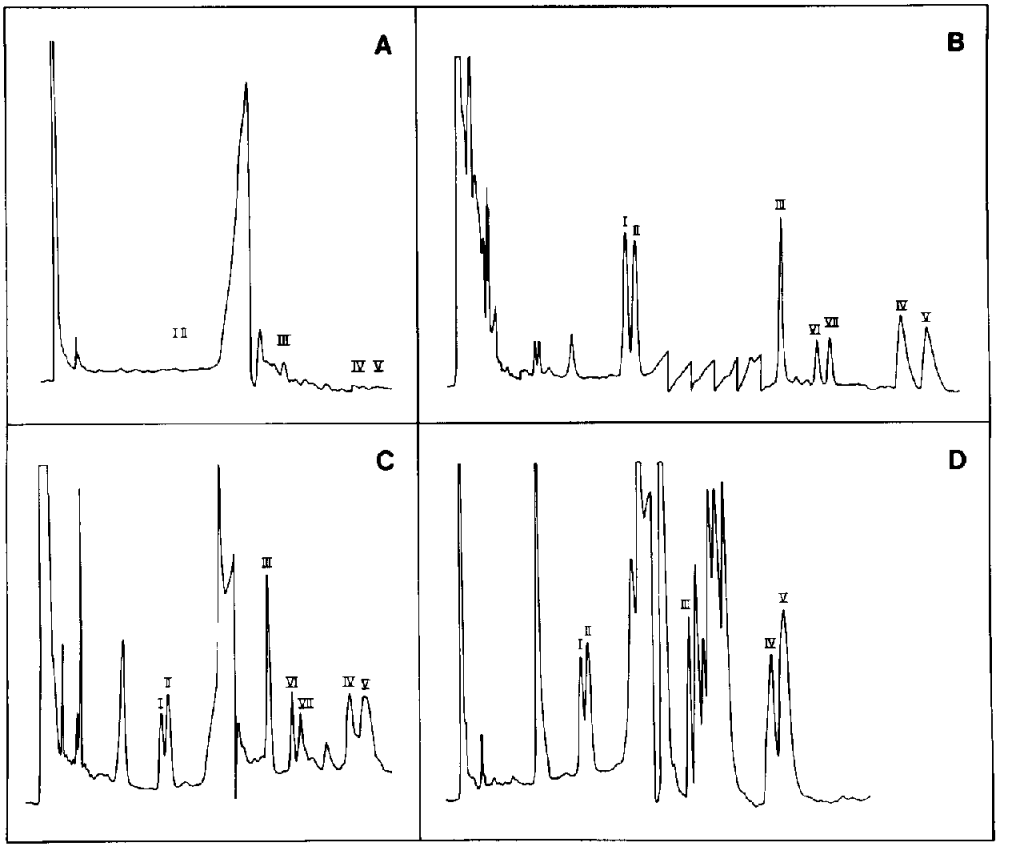

Figure 2
Chromatograms from the analysis of serum samples containing glutethimide and 4-hydroxyglutethimide where I = (*R*)-(+)-glutethimide; II = (*S*)-(−)-glutethimide; III = internal standard; IV = (1)-4-hydroxyglutethimide; V = (2)-4-hydroxyglutethimide; VI and VII = diastereomeric 4-hydroxyglutethimides. Chromatogram A = blank plasma, detector autozeroed after completion of gradient; Chromatogram B = blank plasma spiked with racemic glutethimide (10 μg/ml) and racemic 4-hydroxyglutethimide (12.5 μg/ml), detector repeatedly autozeroed during the gradient; Chromatogram C = serum from a patient suffering from glutethimide intoxication; Chromatogram D = urine sample from a patient suffering from glutethimide intoxication.

analytical method is lengthy it does enable the resolution of the enantiomers of glutethimide (compounds I and II, k' = 7.46 and 7.91, respectively, α = 1.06) and the enantiomers of 4-hydroxyglutethimide (compounds IV and V, k' = 23.0 and 23.9, respectively, α = 1.04) in a single chromatographic run. The method is able to detect concentrations as low as 0.5 μg/ml of each of these compounds. It is interesting to note that the method is able to detect an additional pair of compounds (compounds VI and VII). These peaks were present in the 4-hydroxyglutethimide standard and, since this compound is diastereomeric, it is assumed that peaks VI and VII correspond to the resolution of the diastereomers of 4-hydroxyglutethimide, present as a minor component. These peaks were also detected in the serum of one of the patients suffering from glutethimide intoxication (Fig. 2C). It is possible that the same peaks were present in the urine of this patient, however, interfering peaks make a positive identification difficult (Fig. 2D).

Based on previous work in the authors' laboratory [7], the first glutethimide peak was identified as the (*R*)-(+)-enantiomer. Since sufficient quantities of the 4-hydroxyglutethimide enantiomers were unavailable it was not possible to assign the enantiomeric elution order for peaks IV and V. Accordingly they were designated (1)-4-hydroxy- and (2)-4-hydroxyglutethimide, respectively. However, chiral recognition on the Pirkle-type HPLC–CSPs has been shown to be consistent throughout a closely related series of compounds [10, 11]. This property of the HPLC–CSP suggests that peak IV corresponds to the diastereomer arising from (*R*)-(+)-glutethimide and that peak V corresponds to (*S*)-(−)-glutethimide.

Serial serum samples from three patients with glutethimide intoxication and from one volunteer subject were analyzed using this method. Representative serum time–concentration curves are presented in Figs 3 and 4. The samples used in this study had been analyzed previously [2–4, 6] and the values obtained with this method were consistent with the earlier results.

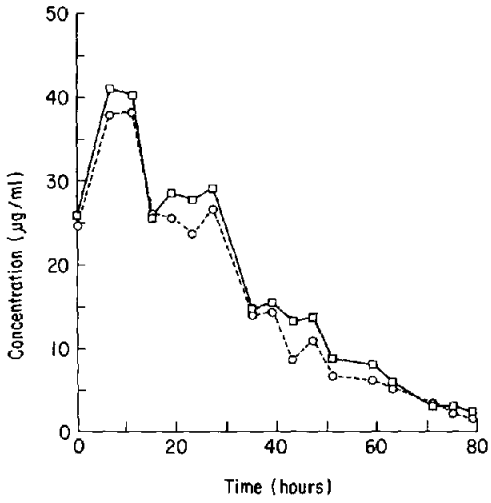

Figure 3
The glutethimide serum time–concentration curve obtained from a patient suffering from glutethimide intoxication. □——□ = (*R*)-(+)-glutethimide; ○- - -○ = (*S*)-(−)-glutethimide.

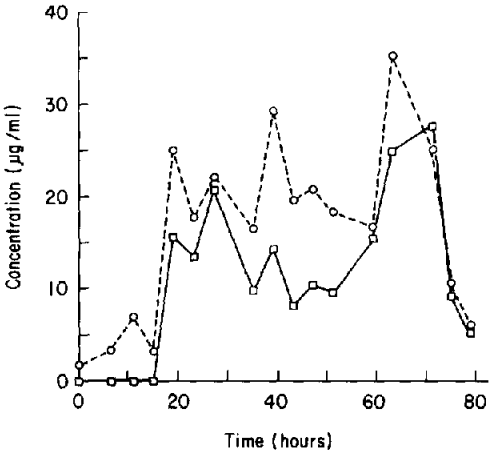

Figure 4
The 4-hydroxyglutethimide serum time–concentration curve obtained from a patient suffering from glutethimide intoxication. □——□ = (1)-4-hydroxyglutethimide; ○- - -○ = (2)-4-hydroxyglutethimide.

Conclusion

A method is presented in this report that is shown to be applicable to the study of the pharmacokinetics of glutethimide and its 4-hydroxmetabolite. Also it is demonstrated that the Pirkle-type HPLC–CSPs can be used with gradient elution thus enabling the simultaneous separation of compounds of different relative polarities.

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