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Note

Quantitative analysis of vigabatrin in plasma and urine by reversed-phase high-performance liquid chromatography

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Vigabatrin (4-amino-5-hexenoic acid, GVG) is a catalytic inhibitor of brain γ-aminobutyric acid (GABA) transaminase [1]. Consequently, when GVG is administered to laboratory animals it produces an increase in brain GABA concentrations [2, 3]. This is believed to be effective in the treatment of neurological and psychiatric disorders associated with GABA deficiencies. Preliminary trials have proven this to be particularly true in cases of epilepsy and tardive dyskinesia [4, 5].

In order to carry out pharmacokinetic and bioavailability studies, it was necessary to develop a relatively simple quantitative analytical method for GVG in plasma and urine. A previous method illustrated the possibility of analyzing GVG by using an amino acid analyzer with microcolumns [6]. This is a time-consuming method requiring regeneration of the column after each analysis. Haegele and Schoun [7] have developed a gas chromatographic—mass spectrometric (GC—MS) method which uses a chiral capillary column for the analysis of the *R*- and *S*-enantiomers of GVG in plasma, urine and cerebrospinal fluid (CSF). The method is somewhat complex, requiring double derivatization and does not lend itself to a large number of samples at one time. However, it is highly selective and sensitive. Methods for analysis of GABA, which are potentially applicable to GVG, include ion-exchange high-performance liquid chromatography (HPLC) with post-column derivatization and fluorescence detection [8, 9]. These are sensitive methods, measuring picomoles of GABA in CSF, but requiring dedicated and somewhat complex instrumentation. Griesmann et al. [10] report a reversed-phase HPLC method for detection of Dns-derivatized GABA in brain tissue. The method involves a tedious series of

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reactions and extractions and does not emphasize accurate quantitation.

This paper describes a reversed-phase HPLC method for accurate and precise determination of GVG in plasma and urine. It functions on readily available equipment, permitting the analysis of many samples on a daily basis. The method is statistically evaluated and applied to a preliminary pharmacokinetic study in dogs.

EXPERIMENTAL

Reagents

Glass-distilled acetonitrile, dioxane, diethyl ether and ethyl acetate were purchased from Burdick & Jackson Labs. (Muskegon, MI, U.S.A.) Reagent-grade boric acid, sodium borate and phosphoric acid were purchased from J.T. Baker (Phillipsburg, NJ, U.S.A.). Copper(II) chloride and Dns chloride were purchased from Aldrich (Milwaukee, WI, U.S.A.). Glass-distilled water was used in all aqueous preparations. GVG and γ-phenyl GABA (γ-aminobenzenebutanoic acid), the internal standard, were synthesized by Merrell Dow Research Institute (Cincinnati, OH, U.S.A.).

Instrumentation

Analyses were performed on an HPLC system consisting of Waters Assoc. (Milford, MA, U.S.A.) Model 6000A solvent delivery system and WISP Model 710B auto-injector. The fluorometric detector was a Kratos Model FS 970 (Schoeffel Instrument Division, Westwood, NJ, U.S.A.). The excitation wavelength was 345 nm and a 418-nm cut-off filter was used on the emission side. Chromatography was on a 25 cm × 4.6 mm I.D. DuPont (Wilmington, DE, U.S.A.) Zorbax C_8 column of 6 μm particle size. The mobile phase was acetonitrile—dioxane—0.5 *M* orthophosphoric acid (35:15:50) with a flow-rate of 1.0 ml/min at room temperature.

Standard solutions

A stock standard solution for the analysis of plasma samples was prepared by dissolving GVG in water (1 mg/ml). A 5-ml aliquot of this was diluted to 25 ml with either human or dog control plasma, making a working standard solution (200 μg/ml) from which six other standards ranging from 150 to 5 μg/ml were made by dilution.

The urine standards, varying in concentration from 4 to 0.02 mg/ml were prepared by dilution from a solution of 10 mg/ml of GVG in control urine.

γ-Phenyl GABA, the internal standard (I.S.), was prepared in a water solution at 0.4 mg/ml.

Plasma

Standard and sample plasma (100 μl) were placed in 100 × 13 mm screw-cap test tubes to which were added 20 μl of the internal standard solution. To this were added 200 μl of acetonitrile followed by 100 μl of 0.03 *M* copper chloride solution. After centrifugation at approximately 800 *g* for 15 min, the supernatant was transferred to a 100 × 13 mm screw-cap test tube. To this were added 200 μl of borate buffer (25 ml of 0.2 *M* boric acid plus 20 ml of 0.05 *M*

sodium borate diluted to 100 ml, pH = 8.45 ± 0.05) and 200 μl of Dns chloride solution (2 mg/ml in acetonitrile). The reaction mixture was vortexed for 5 sec and placed in a 50°C water bath for 15 min. After cooling to room temperature the reaction mixture was extracted with 1 ml of diethyl ether which was subsequently discarded. The aqueous phase was then extracted with 1 ml of ethyl acetate. The ethyl acetate was transferred to a 100 × 13 mm screw-cap test tube and washed with 1 ml of water. After transferring to a dry 100 × 13 mm test tube, the ethyl acetate was evaporated to dryness in a 35°C water bath under nitrogen. The residue was dissolved in 2—4 ml of mobile phase and 50—100 μl were injected on the HPLC column.

Urine

The urine analysis was performed on 10 μl of sample to which were added 100 μl of water, 20 μl of internal standard solution (0.4 mg/ml), followed by 200 μl of acetonitrile and 100 μl of 0.015 *M* copper chloride solution. The mixture was vortexed for 5 sec before the addition of 200 μl of borate buffer (50 ml of 0.4 *M* boric acid plus 20 ml of 0.125 *M* sodium borate diluted to 100 ml, pH = 8.05 ± 0.05) and 200 μl of Dns chloride solution (2 mg/ml in acetonitrile). The reaction mixture was heated at 50°C for 15 min in a water bath. The extraction procedure and ensuing sample preparation for HPLC injection were exactly the same as that described for plasma.

Calculation

Data were analyzed to give the peak area ratio of GVG to internal standard. Values for the samples were determined from the daily standard calibration curve which was calculated by linear regression.

RESULTS AND DISCUSSION

The copper(II) ion complexes with the endogenous α-amino acids [11] which prevents their Dns derivatization. This greatly enhances the specificity of the assay as well as simplifying chromatographic conditions, resulting in a relatively clean chromatogram. The Dns derivatization procedure was developed from conditions reported by Bayer et al. [12] and Tapuhi et al. [13].

Fig. 1A is a typical chromatogram of a plasma standard sample containing 40 μg/ml GVG and 80 μg/ml internal standard. The Dns derivative of GVG elutes at 8.8 min and the internal standard at 13.5 min. Fig. 1B shows the chromatogram of a blank plasma sample and indicates that the region of GVG is free from extraneous interference. The chromatograms of urine extracts are essentially identical to that of plasma. The only difference is that the baseline is flatter with fewer small peaks from endogenous material.

The method for plasma is linear over the concentration range 5—200 μg/ml with a minimum detectable limit of ca. 0.5 μg/ml. In urine GVG is linear from 20 to 4000 μg/ml with a minimum detectable limit of ca. 10 μg/ml.

For a validation of the analysis of GVG in plasma, twelve unknown samples at six different concentrations, making a total of seventy-two samples, were prepared. The samples were assayed in groups of twelve on six different days. Each group contained the six different concentrations in duplicate. They

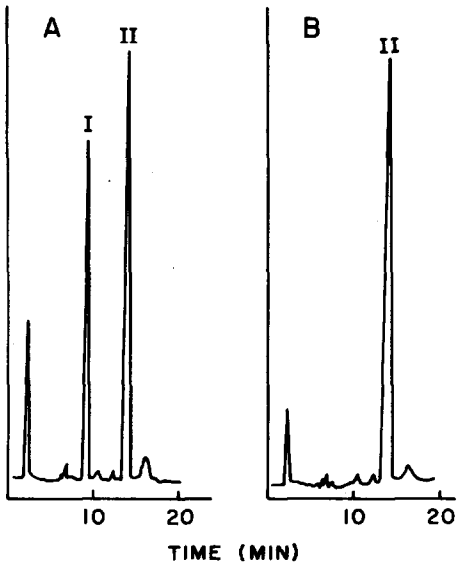

Fig. 1. HPLC profiles obtained from analysis of (A) a plasma sample containing approximately 40 μg/ml GVG and 80 μg/ml internal standard and (B) a blank plasma sample containing internal standard. Peaks: I = GVG; II = internal standard.

TABLE I

RESULTS OF VALIDATION STUDY FOR THE ANALYSIS OF VIGABATRIN IN PLASMA

Theoretical concentration (μg/ml)	Experimental concentration (mean ± S.D., n = 12) (μg/ml)	C.V. (%)
183.5	186.9 ± 5.9*	3.2
116.5	111.6 ± 7.4	6.6
68.0	65.7 ± 3.5	5.3
20.4	20.3 ± 1.5	7.5
4.08	4.1 ± 0.38	9.2
0	0	—

*n = 11.

were compared each day to two standard curves of eight points each including a blank. The mean value of the twelve determinations for each unknown ranged from 95.8% to 101.8% of the theoretical value. The coefficient of variation (C.V.) ranged from 3.2% for the highest concentration of 183.5 μg/ml to 9.2% for the lowest concentration of 4.08 μg/ml. These data are presented in Table I.

Since the urine samples are assayed by essentially the same procedure as the plasma samples, a comprehensive validation of the method was not repeated in urine. However, the mean correlation coefficient of five standard curves in urine run in duplicate was 0.9984 (S.D. = 0.0012, C.V. = 0.12%). There are two differences between the methods for plasma and urine. Because there is much less protein and amino acids in urine, one half the concentration of copper(II) chloride is used for urine as compared to plasma. Because the pH of urine

is usually lower than that of plasma and the salt concentration can be higher, the borate buffer added prior to Dns derivatization is stronger and the pH is 8.05 instead of 8.45. The apparent pH of the urine or plasma sample mixtures prior to the addition of Dns chloride is comparable (urine: pH = 9.0, plasma: pH = 9.1) and the proportion of water to acetonitrile is the same. These are the critical conditions for derivatization and extraction of GVG and internal standard.

It is anticipated that GVG will be excreted rapidly and mostly unchanged; therefore, the urine sample size was reduced compared to plasma and the concentration range of the standard curve was expanded.

Application to preliminary pharmacokinetic study in dogs

A preliminary pharmacokinetic study of GVG was carried out in dogs in order to determine the adequacy of the analytical method regarding plasma levels after dosing.

Two female beagle hounds were given 50 mg/kg GVG in a 5% solution. This was thought to approximate the probable dose that will be administered to man. One dog was dosed intravenously and the other orally. A sample of blood was drawn just prior to dosing and at 0.083, 0.166, 0.25, 0.5, 1, 2, 3, 4, 5, 6, 7 and 8 h after dosing. The blood was drawn by syringe, then added to lithium heparin tubes (Venoject T-200 SLH). It was centrifuged and the plasma was frozen until it was analyzed. Each sample was analyzed in duplicate. The assay results are listed in Table II. The average values were plotted as concentration of GVG (μg/ml) versus time (h) (Fig. 2). The highest plasma concentration of 138 μg/ml for the intravenous dosing was observed at 5 min which was the first sample after dosing. The oral dose peak concentration of 77 μg/ml occurred at

TABLE II

RESULTS OF DUPLICATE ANALYSIS OF GVG IN DOG PLASMA AFTER INTRAVENOUS AND ORAL DOSING OF FEMALE BEAGLE HOUNDS AT 50 mg/kg

Time (h)	Intravenous dosing (dog No. 81-103) (μg/ml)			Oral dosing (dog No. 81-104) (μg/ml)		
	1	2	Average	1	2	Average
Pre-dose	0	0	0	0	0	0
0.083	144.4	133.3	138.3	0	0	0
0.166	126.4	104.5	115.5	7.3	4.0	5.65
0.25	116.3	91.5	103.9	13.9	15.2	14.5
0.5	79.3	69.7	74.5	58.8	66.2	62.5
1	54.0	52.8	53.4	72.7	81.7	77.2
2	23.5	—	23.4	41.4	35.7	38.4
3	17.5	15.5	16.5	19.8	21.9	20.8
4	11.6	11.9	11.7	19.0	19.4	19.2
5	10.4	9.1	9.7	13.2	10.3	11.8
6	6.8	8.4	7.6	12.4	7.2	9.8
7	3.7	5.5	4.6	11.0	3.7	7.4
8	3.0	5.0	4.0	5.9	3.4	4.6
Trapezoidal AUC (μg-min/ml)			10 585			11 644

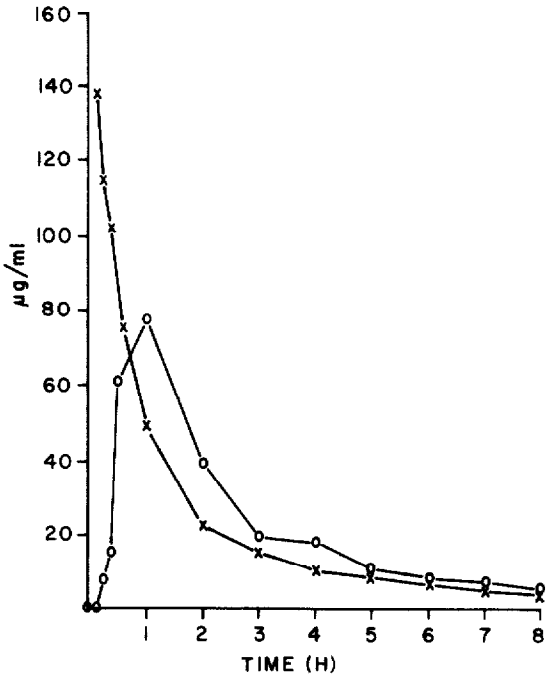

Fig. 2. Concentration of GVG in dog plasma as a function of time after an oral (○) and intravenous (×) dose of 50 mg/kg.

1 h. After 8 h the plasma concentration in both intravenously and orally dosed dogs dropped to approximately 5 µg/ml.

Since the intravenous and oral data (Table II) were obtained from different dogs, no calculations could be done to relate the two routes of dosing. The area under the curve (AUC) values (Table II) indicate, however, that oral absorption was probably substantial if not complete for GVG.

CONCLUSION

The quantitative method described in this report of GVG in plasma and urine is selective and shows a high degree of accuracy and precision. As the preliminary pharmacokinetic study in dogs indicates, it is adequate for full scale bioavailability and pharmacokintic studies.

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