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Automated microanalysis of gabapentin in human serum by high-performance liquid chromatography with fluorometric detection

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Abstract

An automated high-performance liquid chromatographic method for the determination of gabapentin, 1-(aminomethyl)cyclohexaneacetic acid, in serum is described. The procedure involves protein precipitation with methanol followed by using a robotized derivatization with *o*-phthaldialdehyde reagent and automated high-performance liquid chromatography. The analog of gabapentin, 1-(aminomethyl)cycloheptaneacetic acid, was used as the internal standard. Blank serum was fortified with gabapentin (0.1–10.0 μg/ml) and internal standard. Separation was achieved on a Waters 5-μm reversed-phase column (10 cm×4.6 mm) with mobile phase consisting of 0.02 *M* phosphate buffer (pH 4.5)–acetonitrile (50:50, v/v). Eluents were monitored by fluorescence spectroscopy with excitation and emission wavelengths of 230 and 420 nm, respectively. The calibration curve for gabapentin in serum was linear (r=0.999) over the concentration range 0.1–10.0 μg/ml. The inter- and intraassay variations for three different gabapentin concentrations were ≤10% throughout. The lower limit of quantitation was found to be 0.1 μg/ml. Chromatography was unaffected by a range of commonly employed antiepileptic drugs or selected amino acids. © 1999 Elsevier Science B.V. All rights reserved.

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1. Introduction

Gabapentin (GBP) is a new antiepileptic drug that has been approved for the treatment of partial seizures. Whereas GBP is structurally and functionally related gamma-amino-*n*-butyric acid (GABA), the mode of action of GBP is not fully understood [1]. It does not interact with GABA receptors, and it does not inhibit GABA uptake or degradation. It is believed to be related to its ability to enhance the activity of GABA. GBP is rapidly absorbed, and its peak plasma concentrations could occur at approximately 45 min following an oral dose. GBP is not metabolized by the liver, does not induce hepatic enzymes, is not protein bound, and is free of major interactions [2]. Because GBP is new, the role of therapeutic monitoring in the management of patients with epilepsy is unknown [3]. Therefore, measuring GBP serum concentrations is useful in assessing compliance and evaluating risks of toxicity.

Numerous methods have been published describing the measurement of GBP [4–10] using gas chromatography (GC), capillary electrophoresis (CE), or high-performance liquid chromatography (HPLC). The GC and the CE assays are likely to be

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limited by the routine availability of these techniques. The earlier HPLC method used UV photometric detection after precolumn derivatization with 2,4,6-trinitrobenzenesulphonic acid. This method appeared to be hampered by a multistep derivatization and extraction. The more recent methods described the fluorometric detection with use of HPLC gradient system. These methods are somewhat complicated and require long chromatographic runs (>20 min). We have developed an automated, simple, and rapid method for the determination of GBP in serum by HPLC with fluorometric detection. By using fluorometric detection, GBP can be determined more simply and sensitively.

2. Experimental

2.1. Materials and reagents

GBP and the internal standard, gamma-phenyl GABA, were obtained from Johnson Pharmaceutical Research Institute (Spring House, PA, USA). Dade immunoassay controls comprehensive trilevel were from Dade (Miami, FL, USA). Acetonitrile and methanol, both of high-purity quality were obtained from Burdick and Jackson (Muskegon, MI, USA). *o*-Phthaldialdehyde (OPA) reagent solution containing 2-mercaptoethanol (MCE) was from Sigma (St. Louis, MO, USA). Water was deionized and purified by a Milli-Q Millipore purification system (Millipore, Marlborough, MA, USA). Sodium hydroxide was certified ACS grade from VWR Scientific (West Chester, PA, USA). Phosphoric acid 85% of HPLC grade was from Fisher (Fair Lawn, NJ, USA). Potassium phosphate monobasic of HPLC grade was from EM Science (Gibbstown, NJ, USA).

2.2. Standards

An internal standard solution of 1 μg/ml and a GBP stock solution of 1 mg/ml were prepared by accurately weighing 10 mg each of the internal standard and GBP and diluting with the appropriate volume of 50% methanol in water (v/v). A series of standard A and control A solutions was then prepared with the appropriate volume of 50% methanol in water (v/v). Two different pools of patient serum used to construct 10 ml each of the standard B and control B solutions were added to the appropriate volumes of standard A and control A solutions. The series of standard B solutions thus have final concentrations of 0.1, 0.3, 0.7, 1, 2, 3, 5 and 10 μg/ml GBP and the control B solutions have final concentrations of 0.5, 4, and 7 μg/ml GBP. The standard and control samples were stored in 1.8-ml polypropylene tubes (Sarstedt, Newton, NC, USA) at −85°C and used throughout the study.

2.3. Sample preparation

An aliquot of 50 μl of each human serum sample, blank serum, or quality control sample was placed in a 1.5-ml polypropylene tube. The following were added to each tube: 100 μl of internal standard solution and 500 μl of methanol. The tube was vortexed for 30 s on a mechanical vortexer (VWR Scientific Products Multi-Tube Vortexer, Cincinnati, OH, USA), and centrifuged (IEC Centra-8R centrifuge, International Equipment, Needham Hts, MA, USA) for 10 min at 2000 *g* and 5°C. The resulting supernatant was separated from the precipitate.

2.4. Derivatization

A 100-μl aliquot of the supernatant was transferred to an autosampler glass vial (marked sample vial). By using the preparation program in the TSP AS3000 (Thermo Separation Products, Freemont, CA, USA), the following procedures were controlled automatically (see Appendix A). An aliquot of 100 μl of OPA reagent solution was added to the above vial and mixed for 30 s. The reaction mixture was then mixed with an aliquot of 100 μl of 0.5 *M* perchloric acid for 10 s prior to injection of 20 μl onto the liquid chromatograph.

2.5. HPLC

The high-performance liquid chromatograph used in this assay consisted of a P4000 quaternary gradient pump, an AS3000 variable-loop autosampler, a Spectra System FL3000 fluorescence detector, and an IBM PS/277486DX2 computer/controller with PC1000 software (Thermo Separation Products). A reversed-phase Nova-pak C_{18}, Radial-Pak cartridge

(4 μ, 8 mm×10 cm), held by a radial compression module (Millipore–Waters, Milford, MA, USA) as the stationary phase is used. A PRP-1 guard column is used to protect the analytical column. The mobile phase for the isocratic elution of GBP was prepared as follows: monobasic potassium phosphate (2.7 g) are added to 500 ml of water and 500 ml of acetonitrile. The flow-rate was 1.5 ml/min. The HPLC column temperature was 40°C. The AS3000 injector is set at needle height of 1.5 mm and injection volume of 20 μl for each sample. The excitation and emission wavelengths were 230 nm and 420 nm, respectively, with slit widths set at 15 and 20 nm, respectively.

2.6. Quantification

Peak area and peak height measurements for each calibrator, control, and sample were obtained by using the TSP PC1000 software. The peak area ratios of GBP/internal standard were used to obtain a least squares linear regression equation, which was used to calculate the GBP concentrations of the frozen quality control samples and patient samples.

3. Results and discussion

Chromatograms of serum with and without GBP and the internal standard are shown in Fig. 1. The retention times for GBP and the internal standard are 6.4 and 7.9 min, respectively. No significant interfering peaks appear in the chromatogram of blank serum and the two compounds are well separated. When compared to the recently reported HPLC methods of Ratnaraj and Patsalos [9] and Wad and Kramer [10] for the measurement of GBP in plasma, this assay proved to be both more rapid and simple. In contrast to the gradient elution used in these methods, we applied an isocratic elution method. The duration of our chromatographic run is 10 min, compared favorably to their long chromatographic runs (more than 20 min).

Dade quality controls were applied to investigate the potential interference in GBP assay. The quality controls, supplied as lyophilized products from human blood, consist of highly purified chemicals and biochemicals (see Table 1). The low and high

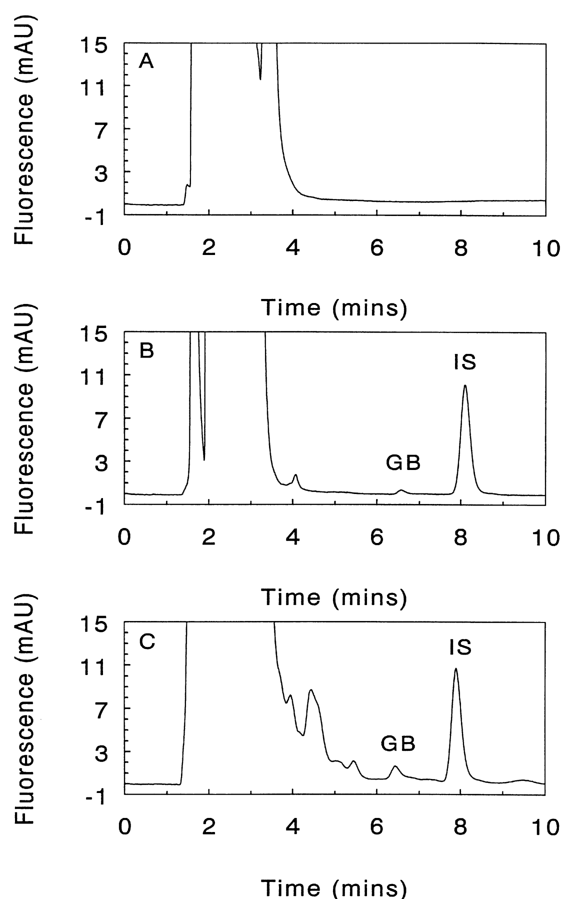

Fig. 1. Typical chromatograms highlighting gabapentin (GBP) and internal standard (I.S.) peaks in profiles of (A) blank human serum, (B) human serum spiked with 0.1 μg/ml GBP and 2 μg/ml I.S., and (C) a clinical sample containing 0.39 μg/ml GBP and 2 μg/ml I.S.

controls were extracted according to the proposed method and the extracts were analyzed. The values assigned to each constituent in Table 1 were approximated according to the values provided in manufacturer's manual and the extraction efficiency of the proposed method. The resulting chromatograms of low and high Dade controls showed no interfering peaks.

A series of standards containing 0.1, 0.3, 1, 5 and 10 μg/ml GBP in human serum was used to construct standard curves which was run on each of five analysis days. Calibrations were linear between 0.1 and 10 μg/ml. The procedure was reproducible from day to day with coefficients of variation (CV%)

Table 1
Quality control materials tested for potential interference in GBP assay; when these analytes were evaluated, no interfering peaks were observed

Material	Approximate amount injected	
	Low, ng	High, ng
Acetaminophen	20	155
Aldosterone	0.00005	0.0005
Alpha-fetoprotein	0.005	0.35
Amikacin	5	30
Carbamazepine	3.5	15
Carcinoembryonic antigen	0.005	0.1
Cortisol	4	30
Digoxin	0.001	0.0035
Disopyramide	1.5	6
Estradiol	0.00015	0.001
Estriol	0.05	0.25
Ethosuximide	35	120
Ferritin	0.0125	0.065
Folate	0.0035	0.0125
Free T_3	0.000005	0.000025
Free T_4	0.0005	0.005
Gentamicin	1	7.5
Human growth hormone	0.0035	0.01
Lidocaine	1.5	7.5
N-acetylprocainamide	5	30
Phenobarbital	15	50
Phenytoin	10	32.5
Primidone	3.5	20
Procainamide	2	15
Progesterone	2.5	15
Prolactin	7.5	85
Prostate-specific antigen	0.5	37.5
Prostatic acid phosphatase	1.5	60
Quinidine	1.5	6
Salicylate	80	600
Testosterone	0.000005	0.0001
Theophylline	7	25
Thyroxine (Total T_4)	0.035	0.15
Tobramycin	1	8.5
Tricyclic antidepressants	0.1	0.5
Triiodothyronine (Total T_3)	0.001	0.004
Valproic acid	35	130
Vancomycin	7	75
Vitamin B_{12}	0.00035	0.00075

<5.5% for the correlation (r=0.999). Based upon the relative standard deviation (measure of precision) of 13.9%, the limit of quantification was 0.1 μg/ml. The limit of detection (LOD) was run by spiking 25 μl of 0.1 μg/ml standard to 50 μl of blank serum along with 100 μl of internal standard. Using a signal-to-noise ratio=3, the LOD of 0.05 μg/ml for GBP was obtained. The LOD compared favorably with the recently reported HPLC assays of Ratnaraj and Patsalos [9] and Wad and Kramer [10]. Frozen quality control samples at 0.5, 4, and 7 μg/ml of GBP in human serum were analyzed each day as part of assay validation. The assay is shown to be accurate (≤3.9% deviation from theoretical concentrations) and reproducible (CVs less than 6%) at each concentration. Between-day precision (Table 2, n=5) studies for three serum controls (0.5, 4, and 7 μg/ml) resulted in CVs of 5.6%, 2.4%, and 4.0%, respectively. The analytical recovery of the three serum controls was 98.9%, 96.7%, and 98.1%, respectively. Within-day precision (Table 2, n=5) studies for three serum controls (0.5, 4, and 7 μg/ml) resulted in CVs of 2.0%, 2.0%, and 0.9%, respectively.

In conclusion, a simple, rapid, robust, and automated microanalytical HPLC method is described for the determination of GBP in patient serum or plasma. This automated micromethod appears to possess sensitivity for the most sensitive pharmacokinetic requirements and be more than adequate for the routine monitoring of GBP concentrations in patients. In addition to excellent sensitivity, it shows excellent specificity, precision, and accuracy for determination of GBP in human serum or plasma. Because only 50 μl sera are required, the method is

Table 2
Within- and between-day precision and accuracy of the assay

	Control 1 (μg/ml)	Control 2 (μg/ml)	Control 3 (μg/ml)
Within-day precision:			
True	0.5	4	7
Mean	0.49	3.87	6.87
SD[a]	0.01	0.08	0.06
CV%	2.0	2.0	0.9
%REC	98.9	96.7	98.1
n	5	5	5
Between-day precision:			
True	0.5	4	7
Mean	0.48	4.01	7.27
S.D.	0.03	0.10	0.29
CV%	5.6	2.4	4.0
%REC	96.2	100.4	103.9
n	5	5	5

[a] SD: standard deviation; CV: coefficient of variation; %REC: % recovery; *n*: number of replicates.

ideally suited for pediatric samples, and sera for other assays would thus be available in the pharmacokinetic studies.

Appendix A. Automated GBP assay procedure programmed on the TSP AS3000 autosampler for the sample preparation and derivatization

Step	Comment
Use Template *1*	Template 1 selected.
Description *GBP assay*	Template 1 is GBP derivatization.
No. of Dilution Cycles *2*	Two dilution cycles is default: one dilution cycle per delivery to Extra Vial (Sam+1).
Heater: *On* Temp: *30*	Turn on heater; set the temperature.
1. Load *200* μl Solv *S-1*	Draw methanol into the solvent-holding loop.
2. Pickup *100* μl *Reag-A*	Sample syringe draws OPA–MCE solution into sample/needle line.
3. Pickup *100* μl *Sample*	Sample syringe draws 100 μl GBP sample into sample/needle line.
4. Add *200* μl to *Sam+1*	Deliver OPA–MCE and Sample into first Extra Vial.
5. Mix for *1.0* min	Mix contents of the first Extra Vial for 1.0 min.
Repeat Dilution Steps	
6. Load *100* μl Solv *S-1*	Draw methanol into the solvent-holding loop.
7. Pick LG *100* μl *Reag-B*	Sample syringe draws 100 μl of 0.5 *M* perchloric acid into sample/needle line.
8. Add *100* μl to *Sam+1*	Deliver perchloric acid into the first Extra Vial.
9. Mix for *0.2* min	Mix contents of the first Extra Vial for 0.2 min.
Overlap Enable *Yes*	Turn on Overlap Enable function.
This procedure requires that *1* Extra Vial(s) be placed immediately after each Sample Vial.	The protocol requires one reaction Vial for vortex mixing OPA–MCE, sample, and perchloric acid.

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