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Simultaneous high-performance liquid chromatographic analysis of pregabalin, gabapentin and vigabatrin in human serum by precolumn derivatization with *o*-phtaldialdehyde and fluorescence detection

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Abstract

A rapid, simple and robust method is presented for the simultaneous determination of the γ-amino-*n*-butyric acid (GABA) derivatives pregabalin (PGB), gabapentin (GBP) and vigabatrin (VGB) in human serum by high-performance liquid chromatography (HPLC). Serum is deproteinized with trichloroacetic acid and aliquots of the supernatant are precolumn derivatized with *o*-phtaldialdehyde (OPA) and 3-mercaptopropionic acid. Separation is achieved on a Alltima 3C18 column using isocratic elution; the drugs are monitored using fluorescence detection. Norvaline is used as an internal standard. Within-day precision (COV; $n = 10$) is 1.2% for PGB (serum concentration 10.0 mg/l), 1.1% for GBP (serum concentration 15.8 mg/l) and 0.3% for VGB (serum concentration 15.5 mg/l). The method is linear up to at least 63 mg/l for PGB, 40 mg/l for GBP and 62 mg/l for VGB. Lower limits of quantitation (LOQ) are 0.13 mg/l for PGB, 0.53 mg/l for GBP and 0.06 mg/l for VGB. No interferences were found from commonly coadministered antiepileptic drugs (AEDs) and from endogenous amino acids.

Experimental design in combination with statistical evaluation (ANOVA) was used to study the robustness of chromatography and sample preparation. The method is very suitable for routine therapeutic drug monitoring and for pharmacokinetic studies.
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Keywords: Anticonvulsants; Pregabalin; Gabapentin; Vigabatrin; Drug analysis; HPLC; Validation; Design of experiments

1. Introduction

The drugs vigabatrin (VGB), gabapentin (GBP) and pregabalin (PGB) are structural analogues of γ-aminobutyric acid (GABA) as shown in Fig. 1. VGB and GBP are used as antiepileptic drugs (AEDs). PGB is recently approved for the treatment of partial seizures in patients with epilepsy and for the treatment of neuropathic pain in Europe [1–3].

The action of VGB is attributed to the irreversible inhibition of the enzyme GABA-transaminase, thus preventing the physiological degradation of GABA in the brain, but a secondary mechanism of a blockade for GABA uptake is also suggested [4]. VGB is supplied as a racemic mixture, but only the (*S*)-(+) enantiomer is pharmacologically active [5].

The action of GBP and PGB is possibly due to their high binding to α_2-δ protein, which is associated with voltage-gated calcium channels. Potent binding at this site reduces calcium influx at nerve terminals and its release of several neurotransmitters, including glutamate, noradrenaline and substance P [3].

These drugs are minimally metabolised and primarily excreted in urine as unchanged drugs. For the analysis of VGB enantiomers in human serum or plasma we reported a method [6], but since the suggested linear relationship between both enantiomers [6,7] determination of the racemate concentration in serum or plasma will be suitable for routine therapeutic drug monitoring. This can be performed by high-performance liquid chromatography (HPLC) and fluorescence detection

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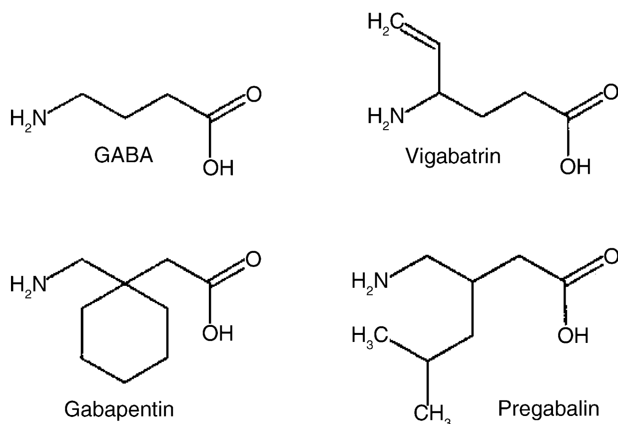

Fig. 1. Chemical structure of GABA and related drugs.

after rapid and simple precolumn derivatization using *o*-phtaldialdehyde (OPA) and 2-mercaptoethanol [8,9]. Besides methods for the analysis of GBP alone [10–12] some papers describe the simultaneous determination of GBP and VGB [13–16]. In this paper, we describe a method for the simultaneous analysis of PGB, VGB and GBP. After precipitation of serum proteins with trichloroacetic acid, an aliquot of the supernatant is derivatized with OPA and 3-mercaptopropionic acid under alkaline conditions. The formed iso-indoles are then separated by reversed phase HPLC under simple isocratic conditions and monitored using fluorescence detection.

The method was validated according to the guidelines of the International Conference on Harmonisation (ICH) [17], including a robustness study by means of experimental design in combination with statistical evaluation of the data (ANOVA). For this purpose we divided the analytical method into two parts: chromatography and sample preparation. The chromatography part was studied by varying six factors using a Plackett–Burman design, the sample preparation part was studied with seven factors and a two-level fractional factorial design.

2. Experimental

2.1. Reagents and standards

(RS)-γ-vinyl-γ-aminobutyric acid (vigabatrin, MRI-71754) was a gift from the Merrell Dow Research Institute (Merrell Dow Pharmaceuticals Limited, Egham, UK). 1-[Aminomethyl] cyclohexane acetic acid (gabapentin) was a gift from Gödecke GmbH AG (Freiburg, Germany) and *S*-[+]-3-isobutyl-γ-aminobutyric acid (pregabalin) was donated by Parke–Davis Pharmaceutical Research (Division of Pfizer Inc., Ann Arbor, MI 48105, USA). Methanol (Lichrosolv), boric acid, sodium hydroxide, hydrochloric acid and trichloroacetic acid (all p.a.) were from Merck (Darmstadt, Germany). Acetonitrile (Chromosolv, Riedel-de Haën), potassium dihydrogen phosphate and dipotassium hydrogen phosphate (both Microselect, Fluka), the derivatizing reagent *o*-phtaldialdehyde (OPA), 2-mercapto-propionic acid (MPA) and the internal standard DL-2-aminopentanoic acid (DL-norvaline) were all obtained from Sigma–Aldrich Chemie B.V. (Zwijndrecht, The Netherlands). Ultra pure water was prepared using the Millipore-Q-plus water purification system (Millipore, Bedford, Massachusetts, USA).

The 0.1 M borate buffer (pH 10.0) was prepared by dissolving 3.1 g of boric acid in approximately 475 ml of water. After adjusting the pH to 10.0 by adding 12 M and 1 M NaOH the volume was adjusted to 500 ml with water.

Stock solution of OPA was prepared by dissolving 100 mg OPA into 3.0 ml of methanol. The derivatizing reagent working solution was prepared by adding 0.3 ml OPA stock solution and 20 μl MPA to 4.0 ml 0.1 M borate buffer (pH 10.0). The storage life of the stock solution is at least 4 weeks at 4–6 °C when protected from light; the working solution is stable for at least 2 weeks when stored at 4–6 °C in the dark.

Stock solutions of VGB, GBP (both 1.5 mg/ml), PGB and internal standard (both 1 mg/ml) were prepared in water and stored at 4–6 °C. The working internal standard solution was prepared by diluting the stock solution with 20 volumes of water prior to use. Calibration serum was prepared in four concentrations by spiking blank new-born calf serum with VGB, GBP (both 7.5, 15.0, 30.0 and 60.0 mg/l) and PGB (5.0, 10.0, 20.0 and 40.0 mg/l).

2.2. Instrumentation

HPLC analysis was carried out on a P4000 quaternary solvent delivery system equipped with an AS3500 autosampler and column oven (Thermo Separation Products Inc., San Jose, CA, USA). Integration and system parameters were controlled by Spectrasystem PC1000 software (Thermo Separation Products). Separation was performed on a home-made reversed phase analytical column (15 cm × 0.46 cm) packed with Alltima 3C18 (Alltech/Applied Science Group, Breda, The Netherlands) kept at a constant temperature of 30 °C. The column eluate was monitored by a FP920 fluorescence detector (Jasco Corporation, Tokyo, Japan) set at an excitation wavelength of 330 nm and an emission wavelength of 450 nm. Detector gain was set at 100. The mobile phase consisted of a mixture of methanol (8.0 vol.%), acetonitrile (17.5 vol.%) and 20 mM phosphate buffer pH 7.0 (74.5 vol.%) and was delivered isocratically at a flow-rate of 0.8 ml/min. Final pH of the mobile phase was 7.50.

2.3. Sample preparation

To 200 μl of serum 50 μl of the internal standard working solution and 100 μl of a 20% solution of trichloroacetic acid in water was added. The mixture was vortexed for 15 s and then centrifuged for 10 min at 2500 × *g*. Ten microliters of the supernatant was transferred to an autosampler microvial (300 μl). Through an automatic sample preparation method

100 μl of borate buffer and 10 μl of OPA working reagent was added. After two mixing cycles, performed by aspirating and dispensing 100 μl of the mixture and after a reaction time of 1 min, 15 μl of the reaction product was injected onto the HPLC system.

3. Results and discussion

3.1. Derivatization

OPA reacts with all primary amino acids under alkaline conditions and in the presence of a thiol. The reaction is complete within 1–2 min and occurs at room temperature [18]. In our method injection cycle was started after 1 min, which resulted in very reproducible results. The fluorescence of the formed iso-indoles starts to decrease after 10–20 min. The use of 3-mercaptopropionic acid compared to mercaptoethanol prolongs the life-time of the derivatives [19].

3.2. Chromatography

In Fig. 2 chromatograms are presented of (a) a blank patients sample and (b) a calibration sample in new-born calf serum. OPA-derivatives of VGB (retention time 4.3 min), the internal standard norvaline (retention time 6.1 min), PGB (retention time 15.0 min) and GBP (retention time 22.1 min) are well separated from the endogenous amino acids. Column life-time, often a problem using OPA-derivatization, is extended by using a small sample injection volume of 15 μl, made possible by a higher sensitivity gain setting of the detector. Replacing the top layer of the column when necessary (broad or split peaks) and reversing the flow direction will prolong the column life-time even more.

3.3. Recovery

Absolute recovery is difficult to calculate in assays including a derivatizaton step but appears to be approximately 100% for the three drugs and was estimated by comparing peak heights in extracted spiked bovine serum with those in standard solutions.

3.4. Repeatability and intermediate precision

Within-day repeatability was determined by subsequent analysis of new-born calf serum spiked with VGB, GBP and PGB at three levels, covering the therapeutic range of the drugs. Results show very low coefficients of variation between 0.26 and 1.27%. Intermediate precision (day-to-day variability) was calculated after analysing control samples at two levels during a 5 months period of routine use of the method by four different technicians on 14 different days. The coefficients of variation ranged from 2.10 to 3.65%. All data are shown in Table 1.

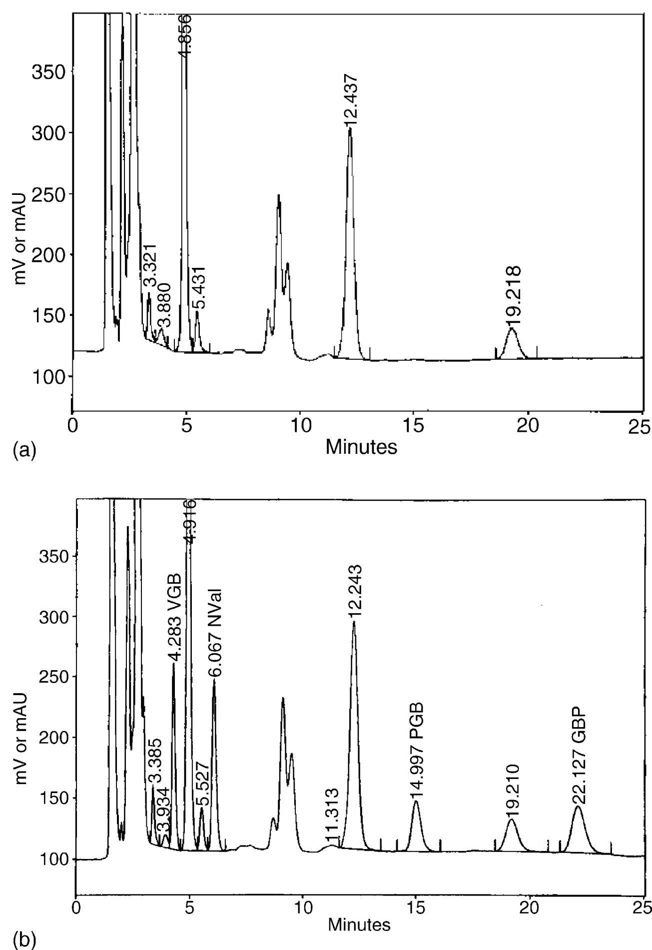

Fig. 2. HPLC-chromatograms of (a) a blank patient sample without internal standard (b) a calibration sample containing 15.51 mg/l of vigabatrin (VGB), 9.99 mg/l of pregabalin (PGB) and 15.75 mg/l of gabapentin (GBP). Retention times are 4.28 min for VGB, 15.00 min for PGB, 22.13 min for GBP and 6.07 min for the internal standard norvaline.

3.5. Linearity, accuracy and sensitivity

For establishment of linearity, accuracy and sensitivity of the method, samples were prepared at seven concentration levels: four as described under 'reagents and standards' and another three by diluting the lowest concentration with blank

Table 1
Method repeatability and intermediate precision

Analyte	Concentration (mg/l)	Coefficient of variation (*n*)	
		Repeatability	Intermediate precision
VGB	7.75	0.81% (6)	3.65% (14)
	15.51	0.26% (10)	
	31.02	0.65% (6)	2.30% (14)
PGB	4.99	1.27% (6)	2.44% (14)
	9.99	1.16% (10)	
	19.98	0.54% (6)	2.32% (14)
GBP	7.88	1.24% (6)	2.10% (14)
	15.75	1.13% (10)	
	31.50	0.72% (6)	2.66% (14)

Table 2
Linearity parameters

Analyte	Linearity range (mg/l)	Intercept	Slope	Correlation coefficient
VGB	0–62	−0.1623	1.0088	0.9998
PGB	0–40	0.0681	0.9831	0.9997
GBP	0–63	0.0621	0.9907	0.9999

Table 3
Accuracy and sensitivity parameters

Analyte	Accuracy		
	Concentration (mg/l)	Coefficient of variation (n = 6) (%)	Accuracy (%)
VGB	62.0	2.41	100.8
	31.0	0.65	100.1
	7.75	0.85	96.8
	1.55	0.83	96.5
	0.78	2.88	83.6
	0.52	1.62	78.0
PGB	40.0	0.55	97.2
	20.0	0.54	101.2
	4.99	1.27	97.3
	1.00	1.24	97.2
	0.50	2.89	99.6
	0.33	3.74	109.3
GBP	63.0	0.72	99.2
	31.5	0.72	99.1
	7.88	1.24	99.6
	1.58	1.32	94.6
	0.79	6.31	103.1
	0.53	4.56	129.1

Analyte	Sensitivity (mg/l) based on			
	(a) Standard deviation blank		(b) Signal-to-noise	
	LOQ	LOD	LOQ	LOD
VGB	0.021	0.008	0.090	0.054
PGB	0.095	0.044	0.173	0.104
GBP	0.773	0.369	0.281	0.168

new-born calf serum up to 15 times. All samples completed with a blank – were analysed at least in three-fold. Linearity parameters are given in Table 2, showing high linearity of the method and covering a range up to twice the upper limit of the therapeutic ranges for VGB and GBP (1–30 mg/l). Plasma levels for PGB at therapeutic doses of 150–600 mg/day were from 0.3 to 14 mg/l [3], sufficiently covered by the tested range.

For sensitivity study the lowest three levels were analysed in six-fold resulting in still low coefficients of variation, however, some unexplained deviations of the target value are observed. In Table 3 these deviations are given under 'accuracy' and presented as a percentage of the target value.

Limit of quantification (LOQ) and limit of detection (LOD) were calculated in two manners: (a) based on the standard deviation of the blank and (b) based on signal-to-noise. In the first case, LOQ and LOD were calculated as 10× and 6× the blanks standard deviation, respectively. In the second case LOQ and LOD were defined as successively 10× and 6× the standard deviation of the noise divided by the signal. See Table 3 for all data.

3.6. *Specificity*

Commonly used antiepileptic drugs and metabolites do not interfere with the method because they do not react with the derivatizing reagent and are not detected under these chromatographic conditions. The following AEDs/metabolites were tested: ethosuximide, primidone, phenobarbital, *N*-desmethylsuximide (metabolite of methsuximide), phenytoin, carbamazepine and its 10,11-epoxide- and 10,11-*trans*-dihydrodiol-metabolites, monohydroxycarbazepine (metabolite of oxcarbazepine), valproic acid, lamotrigine, clobazam and its *N*-desmethyl-metabolite, clonazepam, nitrazepam and diazepam and its *N*-desmethyl-metabolite.

Table 4
Chromatography robustness testing

(a) Selected parameters and their variations	–	0	+
MeOH in mobile phase (vol. %)	7	8	9
MeCN in mobile phase (vol. %)	16.5	17.5	18.5
Molarity phosphate buffer (mM)	15	20	25
Final pH of mobile phase	7.4	7.5	7.6
Flow (ml/min)	0.7	0.8	0.9
Oven temperature (°C)	25	30	35

(b) Plackett–Burman experimental design

Experiment no.	MeOH (%)	MeCN (%)	Phosphate (mM)	Final pH	Flow	OT
1	8.0	17.5	20	7.50	0.8	30
2	9.0	18.5	15	7.60	0.9	35
3	9.0	16.5	25	7.60	0.7	35
4	7.0	16.5	25	7.40	0.9	35
5	9.0	16.5	15	7.40	0.9	27
6	9.0	18.5	25	7.40	0.7	27
7	9.0	16.5	25	7.60	0.9	27
8	7.0	18.5	25	7.40	0.9	35
9	7.0	16.5	15	7.40	0.7	27
10	7.0	16.5	15	7.60	0.7	35
11	7.0	18.5	25	7.60	0.7	27
12	7.0	18.5	15	7.60	0.9	27
13	9.0	18.5	15	7.40	0.7	35
14	8.0	17.5	20	7.50	0.8	30

(c) Responses obtained from experiments

Experiment no.	Vigabatrin				Gabapentin			
	COV	Asymmetry	N/mm	Resolution	COV	Asymmetry	N/mm	Resolution
1	1.6	1.62	36.3	1.27	0.8	1.57	62.3	5.88
2	7.0	1.16	39.6	1.23	0.8	1.54	59.6	6.99
3	0.8	1.63	43.3	1.14	0.3	1.52	68.8	1.23
4	1.0	1.45	42.7	1.33	1.0	1.41	71.8	7.09
5	0.6	1.60	31.0	1.51	0.9	1.42	63.4	3.25
6	1.8	1.61	35.0	1.22	1.3	1.56	63.7	3.94
7	1.4	1.53	37.8	1.12	1.6	1.38	75.7	3.49
8	1.5	1.47	31.1	1.92	0.8	1.41	68.0	5.90
9	1.5	1.59	52.4	1.99	0.5	1.41	77.0	5.88
10	0.6	1.48	46.0	1.26	0.8	1.42	83.1	6.37
11	1.3	1.47	42.1	1.38	4.0	1.44	73.9	5.33
12	5.0	1.23	52.4	1.02	6.9	1.48	64.7	4.28
13	3.7	1.30	47.5	2.06	2.5	1.48	64.6	4.16
14	0.6	1.51	36.3	1.49	0.5	1.44	72.5	6.30

3.7. *Robustness study*

Robustness is an important part of method validation and, as defined by the ICH, it is a measure of the method to remain unaffected by small variations in the specified optimal method parameters and provides an indication of its reliability during normal usage. Experimental design is a useful tool in this kind of studies as it facilitates the investigation of several parameters at the same time while reducing the number of experiments. Preceding the actual testing a selection of essential factors and the levels at which to test them has to be performed and a decision must be made about to consider which responses. Once experiments have been carried out, analysis of variance (ANOVA) can be used to evaluate the results [20–22]. We used a software tool called Essential Regression and Experimental Design for Chemists and Engineers, developed as an add-in for Microsoft Excel® 95/97 and which comes with an electronic book package [21], for setting up experimental designs and for evaluating the results.

Robustness testing was carried out in the pre-validation process of method development, during investigation of accuracy's and precision, and was divided into two parts. Because PGB was included in a later stage of method development, this drug did not participate in this part of the validation process.

Concerning the chromatography part of the method we selected six factors which are likely to be significant in practical use of the method: methanol and acetonitrile content of

Table 5
Sample preparation robustness testing

(a) Selected parameters and their variations	−	0	+
Molarity of borate buffer (mM)	75	100	125
pH of borate buffer	9.5	10.0	10.5
OPA stock volume added to working solution (ml)	0.25	0.3	0.35
MPA volume added to OPA working solution (μl)	15	20	25
TCA concentration of precipitating agent (%)	15	20	25
Sample volume (ml)	0.15	0.20	0.25
Volume of supernatant (μl)	5	10	15

(b) Fractional factorial experimental design and responses

Experiment no.	Molarity of borate	pH borate	OPA	MPA	TCA	Sample volume	Supernatant volume	Responses (COV)	
								VGB	GBP
1	75	9.5	350	25	15	150	15	3.3	1.8
2	100	10.0	300	20	20	200	10	1.0	1.0
3	75	10.5	250	15	25	150	15	3.2	1.2
4	125	9.5	250	15	15	250	15	2.0	2.1
5	75	10.5	350	15	15	250	5	2.2	2.9
6	125	10.5	350	25	25	250	15	1.7	2.2
7	125	9.5	350	15	25	150	5	1.1	1.6
8	75	9.5	250	25	25	250	5	3.7	2.7
9	100	10.0	300	20	20	200	10	1.2	1.3
10	125	10.5	250	25	15	150	5	2.0	4.0

the mobile phase, pH and molarity of the phosphate buffer, flow-rate and column oven temperature. Column type was not taken into account since we use home-made analytical columns packed with Alltima 3C18 from one large homogeneous batch. The selected factors and their variations are shown in Table 4a. A Plackett–Burman screening design allowing the study of up to 11 factors was generated. Together with two centerpoints this resulted in 14 experimental conditions as shown in Table 4b. Due to high environmental temperatures we were forced to use a higher low oven temperature limit of 27 °C instead of the intended 25 °C. In every experiment, we subsequently analysed a calibration serum containing the analytes at their mean therapeutic concentration levels in 10-fold. This resulted in precision data (coefficients of variation, COV) of the analytes that we used as quantitative response. Mean plate numbers, asymmetry factors and resolutions were calculated to evaluate effects on the qualitative response. All responses are presented in Table 4c. In the linear response regression model the acetonitrile content of the mobile phase and the molarity of the phosphate buffer appeared to be two statistically significant factors ($p < 0.05$) concerning both the precision of VGB analysis and the asymmetry of the VGB peak, the first probably being a consequence of the second. Acetonitrile content also appeared to have a statistically significant ($p < 0.1$) effect on the precision and the plate number of GBP. The actual experiments however, never produced a COV greater than 7.0% for VGB and 6.9% for GBP, which is still acceptable.

Robustness of sample preparation was investigated using seven variables with upper and lower limits as shown in Table 5a. This time a fractional factorial design was generated, reducing the number of experiments to 10, including two centerpoints, as shown in Table 5b. Again a calibration serum sample containing the analytes at their mean therapeutic concentration levels was subsequently analysed in 10-fold. Resulting coefficients of variations (Table 5b) were considered as important quantitative response factors. The molarity of the borate buffer was found to be a statistically significant factor ($p < 0.05$) affecting VGB's repeatability, however between the tested limits the coefficient of variation was always below a very acceptable 3.7%.

4. Conclusion

We present a simple, rapid, well validated and robust method for the simultaneous determination of pregabalin, gabapentin and vigabatrin. Experimental design is a very helpful tool in testing robustness of analytical methods during the pre-validation phase. The preceding exploration of its limits is very useful in identifying potential problematic factors and how to control them. The method is in use for routine therapeutic drug monitoring for more than 6 months now, without any problems. The experimental results with respect to linearity, accuracy, precision, specificity and sensitivity demonstrate the reliability of the procedure for its intended application.

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