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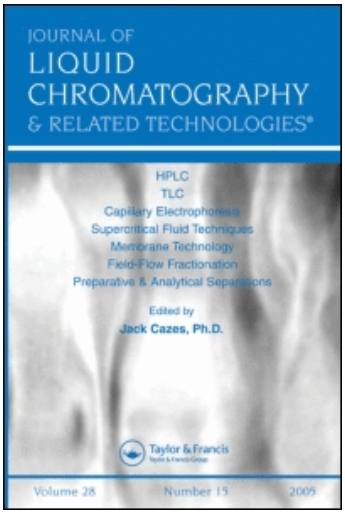

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SIMULTANEOUS HPLC DETERMINATION OF VIGABATRIN AND GABAPENTIN IN SERUM WITH AUTOMATED PRE-INJECTION DERIVATIZATION

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ABSTRACT

A simple and partially automated method for the HPLC determination of the new anticonvulsants vigabatrin and gabapentin in serum samples from epileptic patients is described. The first step in sample pre-treatment is the precipitation of the serum proteins using an excess of acetonitrile to which two internal standards have been added. After centrifugation the supernatant fraction of the samples was placed into an autosampler of an HPLC apparatus which can be programmed with different automatic procedures for pre-injection derivatizations.

The amino acids vigabatrin and gabapentin are derivatized by reactions with ortho-phthaldialdehyde (OPA) and mercaptoethanol. The OPA derivatives are separated on a reversed phase column and measured by fluorimetry. Detection of the gabapentin derivatives requires careful removal of oxygen from the mobile phase by degassing continuously with helium. In this respect the fluorescence signal of vigabatrin derivatives is

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less sensitive to a loss in intensity by the back diffusion of oxygen into the mobile phase after degassing with helium for only a short time.

INTRODUCTION

Vigabatrin (4-aminohex-5-enoic acid, VIGA) is a γ-vinyl derivative of the γ-aminobutyric acid (GABA) and shows its anticonvulsant effect by inhibition of the GABA degrading enzyme GABA transaminase. Thereby a high specificity is combined with a low toxicity.[1]

Gabapentin (1-(aminomethyl) cyclohexane acetic acid, PENTI) was initially developed as a structural analogue to the inhibitory neurotransmitter GABA. Examination has, however, shown that it neither inhibits the uptake of GABA nor does it influence the enzymatic degradation of GABA. An explanation for the anticonvulsant activity of gabapentin is at present still the subject of research.[1] Advantages of therapeutic drug monitoring for the two new anticonvulsant drugs VIGA and PENTI remain still speculative. At least documentation of patient compliance seems to be important in cases refractory to drug therapy. Furthermore the knowledge of serum concentrations may be necessary for studies concerning side effects and efficacy of the drugs.

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The first studies for the analysis of VIGA by HPLC methods were published about 10 years ago.[2-5] Grove et al.[2] carried out protein precipitation in serum and urine samples using trichloroacetic acid and HPLC separation on an ion exchange column in an amino acid analyser. Post column derivatization with ortho-phthaldialdehyde (OPA) was used for fluorimetric detection. Smithers et al.[4] likewise precipitated the proteins from serum and urine samples using an excess of acetonitrile. In order to increase the selectivity of the assay copper chloride was added which should form complexes with the endogenous amino acids. Thus their reaction with dansyl chloride was prevented during the ensuing derivatization. The dansyl derivatives of VIGA and the internal standard (γ-phenyl GABA) were extracted with ethylacetate. After evaporating the solvent off the residues were resolved in mobile phase and injected into a reversed phase column. The separated derivatives were measured by fluorimetric detection.

Chen et al. describe the HPLC separation of the enantiomers of VIGA using UV detection for the diastereomeric derivatives with *tert.*-butyloxy-L-leucine N-hydroxysuccinimide ester.[3] Later on they published an HPLC analysis of VIGA and a primary degradation product in tablets.[5] In this case they carried out a separation on a Partisil® SCX column using UV-detection at

a wavelength of 210 nm. Two further papers on the determination of VIGA in plasma samples using HPLC with fluorimetric detection of OPA derivatives were published in 1991 and 1993. Thereby in both methods the proteins of the plasma samples were precipitated by adding methanol.[6,7]

At least three papers dealing with the HPLC analysis of PENTI have been published. In these papers the same derivatization reagent (2,4,6-trinitrobenzene-sulphonic acid, TNBSA) and the internal standard 1-(aminomethyl) cycloheptane acetic acid (Gö-3609) were used. In the basic study from 1985 of Hengy and Kölle[8] protein precipitation of the plasma samples was carried out using perchloric acid prior to derivatization. The TNBSA products were purified in two liquid liquid extraction steps.

Fraser and MacNeil[9] precipitated serum proteins with acetone and purified the derivatives with acetic acid precipitation. In the third paper by Lensmeyer et al.[10] PENTI and the internal standard were extracted from the serum by a solid phase extraction on a C_{18} cartridge. Derivatization was performed in the methanolic eluate and the reaction products were purified by a further solid phase extraction with a hydrophobic filter membrane (Empore disk® C_{18}).

In the following paper an HPLC method for the simultaneous determination of Vigabatrin and Gabapentin is described. A simple sample pretreatment in which proteins were precipitated with acetonitrile was followed by the automated derivatization of the analytes with the reagent OPA / mercaptoethanol. The reaction products were finally separated on a reversed phase column and measured by fluorimetric detection.

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MATERIALS and METHODS

Apparatus and Reagents

As HPLC system for automated derivatization the 1090 LC with a workstation and a 1046A programmable fluorescence detector from Hewlett-Packard, Waldbronn (Germany), was used. Gradient separations were carried out on a BANsil C_{18} column (5 µm, 250 x 4 mm) from ASMT, Enger (Germany).

Acetonitrile, water, and methanol were of HPLC gradient quality from Riedel-DeHaen, Hannover (Germany). All other reagents used were of analytical reagent grade from Merck, Darmstadt (Germany).

Vigabatrin and the internal standard (ISTD) substance γ-phenyl GABA were obtained from Marion Merrel Dow Research Institute, Cincinnati, Ohio (USA), and Gabapentin and the ISTD Gö-3609 were obtained from Parke Davis, Freiburg (Germany).

The ISTD solution consisted of 100 mg γ-phenyl GABA and 10 mg Gö-3609 dissolved in 500 mL acetonitrile and 500 mLwater.

The borate buffer was made with 15.5 mg boric acid dissolved in 500 mL water and adjusted to pH 9.5 with a concentrated sodium hydroxide solution in water.

The OPA reagent mixture consisted of 100 mg *ortho*-phtaldialdehyde, 9 mL methanol, 1 mL borate buffer, and 100 μL mercapto-ethanol.

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Calibration and Control Samples

For the production of calibration samples stock solutions were made up with 20 mg VIGA and 10 mg PENTI in 100 mL of a mixture of acetonitrile/water (1:1, v/v). With this solvent mixture the analytes and also the ISTD substances can be dissolved easily, whereas acetonitrile or water alone are not equally suitable as solvents for the compounds mentioned. 50 μL portions of the stock solution were added to 500 μL blank serum in 10 mL centrifuge tubes with screw caps. The tubes were closed and frozen at -18°C.

As no controls for the routine determination of VIGA or PENTI are commercially available serum samples from patients with Sabril® (Viga-batrin) or Neurontin® (Gabapentin) medication were pooled. From the pooled sera 500 μL portions were frozen and analysed following the method described in order to check the run-to-run precision of the results.

Extraction and Derivatization

500 μL ISTD solution and 1000 μL acetonitrile were added to 500 μL of patient serum in 10 mL centrifuge tubes (as well as to the calibration and control samples). The tubes were shaken for 5 min in a vortex apparatus. The mixture was centrifuged for 15 min.

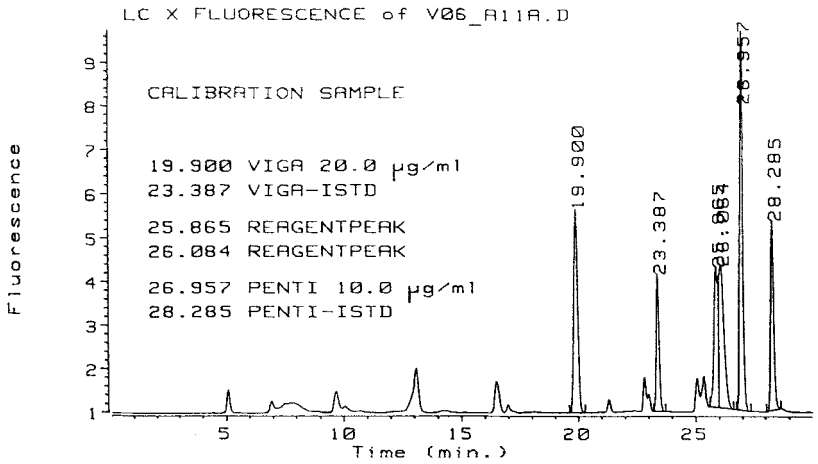

Figure 1. Chromatogram of a calibration sample with 20 µg/mLVIGA and 10 µg/mL PENTI in blank serum

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The clear supernatant fraction was transferred to the derivatization procedure in a special "automated pre-column sample preparation system"[14] which consists of the 1090 LC autosampler and a derivatization program of the HP workstation. The sample extract and the reagents for the OPA derivatization are pipetted up from the different vials by the autosampler and transferred into the injector loop.

After mixing the collected portions by a forward/backward movement of the injector syringe and a delay time for completion of the OPA reaction, the derivatization products are injected onto the analytical column. The derivatization steps are as follows: draw 0 µL from vial 0 (containing acetonitrile/water for flushing the outside of the injection needle), draw 3 µL from vial 1 (containing borate buffer as described above), draw 3 µL from vial 2 (containing the OPA reagent mixture as described above), draw 0 µL from vial 0, draw 6 µL from a sample vial, draw 0 µL from vial 0, draw 3 µL from vial 2, draw 3 µL from vial 1, draw 0 µL from vial 0, draw 2 µL of air, mix 20 cycles with a 10 µL volume of the syringe, wait 1 min, inject.

Parameters of Separation and Detection

Chromatography of the derivatization products was carried out by a gradient elution at 40°C to obtain a baseline separation of the four analytes from endogenous compounds and reagent interferences (see figures 1-4).

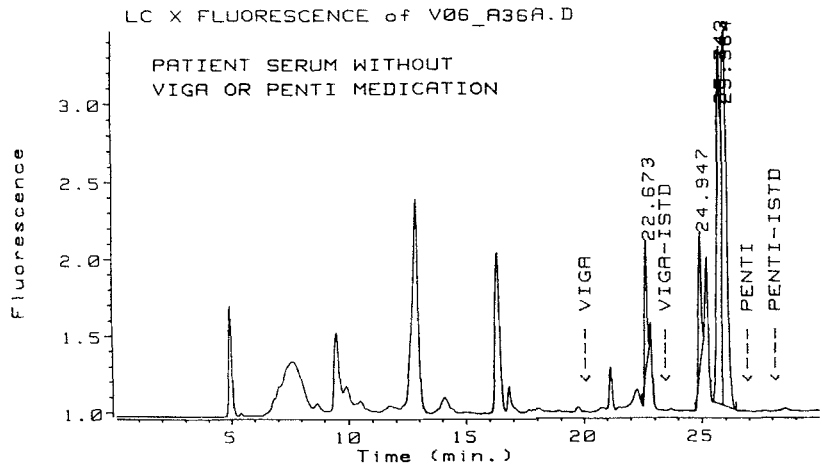

Figure 2. Chromatogram of a serum from an epileptic patient without Sabril® or Neurontin® medication.

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Solvent A for the gradient mixture was 0.1% phosphoric acid (pH 2), acetonitrile, and methanol (8:1:1, v/v/v) and solvent B was acetonitrile and methanol (1:1, v/v). The flow rate was 1.0 mL/min. The gradient profile was as follows: 10% B from min 0 to min 1, from 10% to 70% B from min 1 to min 26, 70% B from min 26 to min 29, and back to 10% B from min 29 to min 29.1, stop run at min 30. Delay time before the next injection was 3 min.

The detection parameters of the fluorescence detector were as follows: Excitation = 235 nm, emission = 435 nm, Pmtgain = 8, lamp frequency 55 Hz, response time = 1000 msec.

RESULTS and DISCUSSION

Extraction Conditions and Sample Amount

As described earlier[11,12] it was observed that a one to one ratio of acetonitrile and the aqueous serum sample was not sufficient to yield a complete deproteinization. With an ISTD solution containing equal amounts of acetonitrile and water and a two-fold volume of acetonitrile added to the serum

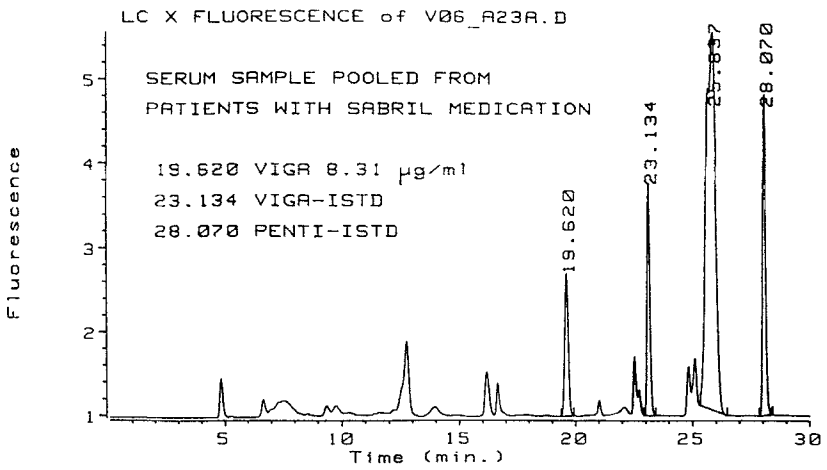

Figure 3. Chromatogram of a patient serum with a VIGA concentration of 8.3 µg/mL.

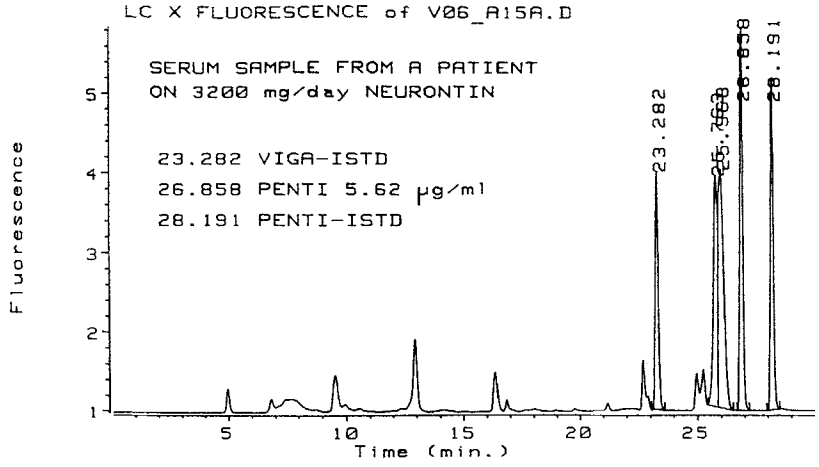

Figure 4. Chromatogram of a patient serum with a PENTI concentration of 5.6 µg/mL.

sample the final amounts of acetonitrile (1250 µL) and the aqueous phase (750 µL) are in a ratio of 5 to 3. With this minimum excess of acetonitrile a complete precipitation of the proteins can be achieved.[13]

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The amounts of the samples, of the ISTD solution and of the precipitation reagent, as given above, were chosen to result in a better handling and a better precision in pipetting the volumes. If smaller sample amounts were obtained from the clinic (e.g. paediatric samples) a reduction of the volumes in the pre-treatment of the patient samples causes no problems if ratios of sample amount and ISTD are the same as with the calibration samples.

From a mixture of 100 µL serum, 100 µL ISTD solution, and 200 µL acetonitrile more than enough of the supernatant fraction is obtained for the derivatization procedure in which only 6 µL of the sample extract are needed for one chromatographic separation. Therefore, if necessary, the volumes in the sample pre-treatment step can be reduced even more to an extent which is limited by the precision of the manual pipetting and the handling of the smaller volumes.

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Chromatographic Conditions

The main problem in the HPLC analysis of drugs which are amino acids consists in separating the analytes and the internal standards from endogenous amino acids which are always present in the serum samples and which also react with OPA. In order to optimise this separation we developed a gradient with a phosphoric acid/acetonitrile/methanol mixture to separate VIGA, PENTI and the two internal standard substances from the other serum constituents (see figures 1-4).

As can be seen from figures 1-4 the gradient separation delivers peaks of VIGA, PENTI, and the two ISTDs at positions in the chromatograms which are not overlapped by the peaks of derivatives of the endogenous substances in the serum (especially figure 2).

Linearity of Detection

In order to check on the linearity of the fluorimetric detection, different amounts of VIGA and PENTI were dissolved in water/acetonitrile (1:1) in a range much higher than normally expected in patient samples and measured as described above. For VIGA, linearity was measured in a range from 0 to 300 µg/mL with a coefficient of correlation (CC) of 0.99988 and a standard error of estimate (SEE) of 1.9094. The slope was 1.006 and the intercept -.519.

For PENTI, linearity was measured in a range from 0 to 300 µg/mL with a CC of 0.99985, a SEE of 1.3271, a slope of 0.9947, and an intercept of 0.238.

Table 1

Recovery of VIGA and PENTI from Serum Samples after Deproteinization

Vigabatrin						
Conc. [μg/mL]	1.05	2.50	10.0	20.0	40.0	Mean
Recovery [%]	94.34	111.2	100.7	97.59	101.1	100.97
Gabapentin						
Conc. [μg/mL]	1.01	4.90	10.1	19.9	39.8	Mean
Recovery [%]	97.78	100.1	100.2	99.60	99.79	99.47

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Recovery of the Extraction Procedure

In order to determine the degree of recovery of the analytes from the serum using the extraction and derivatization methods described above stock solutions containing VIGA and PENTI were added to water as well as to blank serum and analysed (n = 4 for each concentration) in the same way as patient samples.

The results, given in Table 1, show no relevant differences between the results obtained from aqueous or serum samples spiked with VIGA and PENTI. Thus the sample pre-treatment given above yields in a complete recovery of the analytes from serum samples.

Method Precision

Before determining the overall precision of the method described for the analysis of serum samples it must first be checked to what extent the automatic derivatization with the HPLC equipment used for the analysis of VIGA and PENTI contributes to imprecision.

Therefore the derivatization program was carried out twelve times by the autosampler with the same solution of suitable concentrations of the analytes in acetonitrile and water and the deviations of the results were statistically checked.

As seen from the results given in Table 2 the degree to which the apparatus contributes to the imprecision of the automatic derivatization of VIGA and

Table 2

Precision of the HPLC Apparatus for the Automatic OPA Derivatization

	n	Max.	Min	Mean	SD	CV%
VIGA	12	20.00	19.24	19.77	0.2053	1.039
PENTI	12	10.10	10.00	10.07	0.0325	0.323

(SD = standard deviation, CV% = coefficient of variation)

PENTI with OPA is between 1.039% and 0.323% determined as coefficient of variation, if the influence of serum extraction by protein precipitation and the effect of the sample background in the chromatogram due to the serum matrix are omitted.

In order to measure the overall precision of the method described pooled samples from the sera of patients on treatment with Sabril® or Neurontin® were analysed. In each case different concentrations were measured several times on the same day to establish the "within-run" precision.

Likewise the values of pooled samples measured as internal controls over a longer period of time were evaluated in order to establish the "run-to-run" precision. The results in Table 3 show coefficients of variation far below 5% which show that the method described is well suited for routine determinations in clinical laboratory analysis.

Limit of Determination

Normally the range of the determination is related to the "therapeutic range" of the drugs to be analysed. Whereas the detection limit of the method described for the determination of VIGA and PENTI in serum samples might be well below 0.1 μg/mL, a limit of determination was settled with 1.0 μg/mL for Vigabatrin and with 0.5 μg/mL for Gabapentin. These limits meet the practical requirements of the routine analyses of both anticonvulsants.

As shown in table 4 the results of the coefficients of variation of small peaks, even with concentrations just below the determination limits, are tolerable for routine analyses of patient samples.

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Table 3

Within-Run and Run-to-Run Precision for VIGA and PENTI in Serum Samples (conc. μg/mL)

	VIGA "within-run"	PENTI "within-run"
N	20	20
Minimum	8.11	3.65
Maximum	8.53	3.80
Mean value	8.370	3.762
standard deviation	0.0913	0.0307
C.V. (%)	1.091	0.816
	VIGA "run-to-run"	**PENTI "run-to-run"**
N	22	22
Minimum	7.82	3.83
Maximum	8.84	3.99
Mean value	8.425	3.925
standard deviation	0.2097	0.0450
C.V. (%)	2.489	1.147

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Lower concentrations than those calculated in table 4 are hardly of clinical significance for routine analysis, but are possibly of scientific interest. Then it is recommended that in sample pre-treatment a higher effect of concentrating the analytes must be achieved as for example is described by Lensmeyer et al.[10] for the SPE extraction of PENTI from serum samples.

Special Requirements of the OPA Derivatization of PENTI

On developing the analytical procedure with automated OPA derivatization as described above we started with the development of a routine determination of VIGA in patient sera. In the beginning the mobile phase was degassed with helium only intermittently during refill of the solvent bottles in the HPLC apparatus. With this handling an unexpected but steady decrease of the PENTI and PENTI-ISTD concentrations was observed.

After some experiments it became evident that rediffusion of oxygen from the air resulted in a steadily decreasing intensity of the fluorescence signal in the case of both substances, whereas the OPA derivatives of VIGA and VIGA-ISTD were shown to be not sensitive against the oxygen concentration in the mobile

Table 4

Within-run Precision for low Concentrations of VIGA and PENTI in Serum (conc. μg/ml)

	VIGA "within-run"	PENTI "within-run"
N	10	10
Mean value	0.873	0.387
standard deviation	0.0356	0.0071
C.V. (%)	4.082	1.837

phase. With degassing the solvent reservoirs continuously with a very low helium gas flow we overcame this problem. This gives us the possibility to carry out routine determinations of VIGA and PENTI in the same analytical series. In addition we have the option to calculate the PENTI levels as determined using the VIGA-ISTD. This could be of importance in the near future as we were informed that the PENTI-ISTD will not be produced any longer by the manufacturer.

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