were, however, unable to confirm the previously reported association between the 259R allele and lower concentrations of holo-TC in plasma (8, 21). Nevertheless, holo-TC in CSF was reduced in patients with the 259PR and 259RR genotypes, showing that the polymorphism indeed affected holo-TC concentrations in CNS. Holo-TC concentrations in plasma and CSF were highly correlated, which is compatible with the notion that all holo-TC in CSF originates from plasma and needs to pass the blood–brain barrier to enter the CNS. Comparison of the slopes of the linear correlation equations for the homozygous 259RR and 259PP genotypes suggests that holo-TC encoded by the 259R allele crosses the blood–brain barrier less efficiently or is less stable in CSF than 259P-encoded holo-TC. Begley et al. (13) showed that human astrocytes synthetize and secrete TC. Thus, a certain proportion of total TC in CSF most likely originates from within the CNS. One is tempted to speculate that the disrupted correlation between plasma and CSF concentrations of total TC in individuals with the 259PR and 259RR genotypes may reflect up-regulation of TC synthesis within CNS in response to decreased import of B12 across the blood–brain barrier. A similar relationship between vitamin B12 and total TC was seen previously in plasma in which B12 concentrations were inversely correlated with total TC, and total TC was seen previously in plasma in which B12 deficiency or is less stable in CSF than 259P-encoded holo-TC. Bhattacharya et al. (22) showed that human astrocytes synthetize and secrete TC. Thus, a certain proportion of total TC in CSF most likely originates from within the CNS. One is tempted to speculate that the disrupted correlation between plasma and CSF concentrations of total TC in individuals with the 259PR and 259RR genotypes may reflect up-regulation of TC synthesis within CNS in response to decreased import of B12 across the blood–brain barrier. A similar relationship between vitamin B12 and total TC was seen previously in plasma in which B12 concentrations were inversely correlated with total TC, possibly reflecting B12-mediated regulation of expression or clearance of the protein (22).

In conclusion, the TC 259R allele is associated with lower TC concentrations, especially in CSF in patients with Alzheimer disease. We plan to repeat the study in other populations and explore the possible association between the polymorphism and neuropsychiatric symptoms attributable to vitamin B12 deficiency.

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References


Procedure for the Monitoring of Gabapentin with 2,4,6-Trinitrobenzene Sulfonic Acid Derivatization Followed by HPLC with Ultraviolet Detection, JoEtta M. Juenke,1,* Paul I. Brown,1 Gaedaldyn A. McMillin,1,2 and Francis M. Urry1,3 1,2 ARUP Institute for Clinical and Experimental Pathology, ARUP Laboratories Inc, 500 Chipeta Way, Salt Lake City, UT 84108; 2 Department of Pathology, University of Utah Health Sciences Center, Salt Lake City, UT 84108; 4 author for correspondence: fax 801-584-5207, e-mail juenkejm@aruplab.com

Gabapentin is a novel anticonvulsant drug that was introduced in the early 1990s and later approved (1995) for use in the US as an adjunctive treatment of partial seizures with or without secondary generalization in persons >3 years of age. Although structurally similar to γ-aminobutyric acid (GABA), gabapentin does not interact with GABA receptors, nor is it converted to GABA or a GABA agonist (1). Gabapentin is widely studied therapeutically. Its initial and approved use as an adjunctive epileptic therapy has been broadened, with many additional indications. These include treatment for neuropathic pain after spinal cord injury (2–4), posttraumatic stress disorder (5), poststroke pain syndrome (6–8), alcohol withdrawal (9), migraine therapy (10), hot flushes associated with prostate cancer treatment (11), and postoperative pain after cancer surgery (12, 13).
The general mechanism by which gabapentin exerts its anticonvulsant action is unknown. It is not appreciably metabolized in the liver, nor does it induce liver enzymes. It circulates relatively unbound in serum, with a protein bound fraction of ~3%. It has a volume of distribution of ~58 L. Because gabapentin does not bind to protein, it can be removed by hemodialysis if medically necessary. Gabapentin is renally eliminated with an elimination half-life of ~6 h and clearance proportional to creatinine clearance. Impaired renal function substantially decreases the clearance of gabapentin (14). Gabapentin exhibits saturable absorption, making it a nonlinear drug and kinetically less predictable. A dose–response pattern is apparent for plasma gabapentin concentrations and for clinical effects within the dosage range 600–1800 mg/day. Seizure control has not been seen with trough plasma concentrations <2 mg/L. A majority of patients at suggested doses fall within a 2–10 mg/L range. The major side effects of the drug include somnolence, dizziness, ataxia, fatigue, and nystagmus. No serious or irreversible effects occur after overdose. Therefore, monitoring of trough plasma gabapentin concentrations is most useful to establish compliance. In treating chronic pain and addictions, higher trough concentrations of 15–30 mg/L are maintained. Administration of gabapentin does not influence the pharmacokinetics of conventional anticonvulsant drugs, nor are the pharmacokinetics of gabapentin modified by the presence of other anticonvulsant drugs (1).

Gabapentin measurements have traditionally involved a derivatization step. Underivatized it exhibits essentially no ultraviolet absorbance. Several types of analysis have been performed, including spectrofluorometry (15–17) and HPLC with a variety of detection methods, including mass spectrometry and capillary electrophoresis, but most frequently, fluorescent derivatization (18–23). Gas chromatography (GC) has also been used with detection by mass spectrometry (MS) and flame ionization (24, 25). Here we describe a rapid and cost-effective isocratic liquid chromatography method that uses commonly available ultraviolet detection for gabapentin quantification without the need for solid-phase extraction. The assay system included an automated HP1090 liquid chromatograph with autosampler, diode array detector, and a Perkin-Elmer Nelson 1022™ Integrator for result recording. The detector wavelength was set at 335 nm, the absorbance maximum for the 2,4,6-trinitrobenzene sulfonic acid (TNBSA; Pierce Scientific) derivative of gabapentin. The analytes were separated on a 50 × 3.0 mm (i.d.) Luna™ 5-µm phenyl-hexyl column (Phenomenex) with a 4 × 3.0 mm (i.d.) phenyl guard cartridge (Phenomenex) as precolumn. The flow rate was 1.0 mL/min with an approximate backpressure of 5800 kPa at ambient temperature. The mobile phase consisted of acetonitrile–type I water–concentrated glacial acetic acid (500:500:1 by volume). HPLC-grade acetonitrile and acetic acid were purchased from Fisher Scientific. The mobile phase was filtered and degassed before use through a 0.45 µm nylon membrane under reduced pressure.

The internal standard (IS), 3-amino-2-naphtholic acid, was purchased from Aldrich, and gabapentin stock was obtained from Pfizer. The primary stock solutions (100 g/L) of gabapentin and IS were prepared in type I water and methanol, respectively.

Samples were prepared by transferring 0.5 mL of patient sample, positive control, negative control (blank), and calibrators into respective microcentrifuge tubes; 50 µL of IS solution was then added to each tube, followed by 0.7 mL of acetonitrile. The tubes were capped, vortex-mixed for ~30 s, and centrifuged for 5 min at ~7287g. The supernatant was transferred to a clean, screw-top tube, in which it was completely dried down under desiccated air at 50–60 °C. To each tube, we added 0.5 mL of 0.1 mol/L sodium bicarbonate, pH 8.5 (Sigma), and 0.2 mL of freshly prepared derivatization solution. The derivatization solution contained TNBSA prepared daily in 0.1 mol/L sodium bicarbonate (400 µL TNBSA/mL of bicarbonate). The tubes were then tightly capped and incubated at 90 ± 10 °C for 20 min. After incubation, the tubes were centrifuged for 2 min at 1121g. The caps were then removed from the tubes, and the derivatization was stopped by the addition of 0.25 mL of 100 g/L sodium dodecyl sulfate (Sigma) and 0.2 mL of 1 mol/L HCl (Fisher). The solution was transferred to a glass autosampler vial and capped tightly; 40–60 µL was then injected on the column by the autosampler. The ratio of the peak height of the analyte divided by the peak height of the IS was used to quantify the analytes of interest from the calibration curve.

We evaluated the linearity of the method by analyzing in-house C18-filtered blood bank plasma to which gabapentin was added at the following concentrations: 1, 2, 5, 10, 20, 30, 40, 50, and 60 mg/L. This experiment was performed three times over several weeks. Each calibrator was then analyzed in duplicate, and concentrations were determined as the lowest concentration tested that produced a peak twice as great as the baseline noise of a plasma blank. As a result, 1 mg/L was selected as the lower limit of quantification and as the low calibrator for the assay.

Shown in Fig. 1 are chromatograms obtained from a blank plasma (panel A), a representative patient (panel B), and the 10 mg/L calibrator (panel C). The peaks of interest, the IS at 2.473 min and gabapentin at 2.800 min, were followed by four constant derivatization peaks that eluted in the next 3.5 min for a total run time of 6.3 min. After further study (data not shown), the first peak was identified as GABA. The other peaks remain undetermined, but studies have concluded that these peaks are not attributable to the use of sodium dodecyl sulfate. A small side post peak was also seen with the IS when heparinized plasma was used, but it was found to be
consequential in correlation. The peak was not present in EDTA plasma.

To check for specimen interference, we processed 25 deidentified patient samples. Two drugs that potentially will interfere at 335 nm are baclofen (Lioresal®) and tiagabine (Gabatril®), both of which coelute with gabapentin. Baclofen and tiagabine concentrations of 100 and 300 μg/L, respectively, produced a false gabapentin concentration of ~2.6 mg/L (data not shown). Other drugs assayed at therapeutic concentrations that had no interference included carbamazepine and its epoxide and hydroxy metabolites, oxcarbazepine and its mono hydroxylated metabolite, zonisamide, levetiracetam, phenytoin and its metabolites, felbamate, lamotrigine, clonazepam, phenobarbital, primidone, acetaminophen, salicylate, ibuprofen, amitriptyline, nortriptyline, desipramine, doxepin and nordoxepin, imipramine, valproic acid, topiramate, mephenytoin and Nirvanol, amiodarone and desethylamiodarone, methsuximide and normethsuximide, ethotoin, clozapine, and sertraline.

Carryover was studied by injecting a high calibrator (60 mg/L) followed by blank plasma over several runs, over several days. No carryover was observed for the 60 mg/L calibrator.

We compared 30 deidentified patient samples testing positive for gabapentin with another reference laboratory GC-MS assay. The linear regression equation for correlation, where \( y \) is the HPLC method, was: \( y = 1.05x + 0.84 \) mg/L. The 5% slope correlated to no significant difference in patient results and was deemed acceptable.

In conclusion, this report describes a robust assay for the measurement of gabapentin by HPLC with ultraviolet detection that uses TNBSA as a chromogenic derivatization agent. The mechanism of binding to primary amines allows for selectivity toward the analyte of choice. Many coadministered medications either do not have a primary amine site or structurally inhibit binding of the chromophore at the site. The product is stable for at least 48 h at room temperature, whereas derivatives from other methods used are stable for 4–12 h. Use of this chromophore in conjunction with a phenyl-hexyl column in place of the common C18 column enhances selectivity for gabapentin. The phenyl-hexyl column uses hexyl alkyl groups instead of traditional propyl chains, giving it added stability in an acidic mobile phase and high selectivity for aromatic, amine, and polar compounds. Column lifetime generally supercedes 2000 injections, with the precolumn being replaced every other day or ~300 injections and the column washed with acetonitrile during daily instrument maintenance. The extraction process avoids the use of solid-phase extraction, which substantially decreases set-up time. Cost savings are seen in the savings from solid-phase extraction columns, less expensive derivatization reagents, and technologist time compared with our previous GC-MS assay (24). Each step in the extraction causes a change in color, easily identifying each step in the procedure, and allows for quick extraction troubleshooting. Use of the HPLC method reduced our sample reporting time by one-half from the previous GC-MS assay. Although this chromatographic assay is limited by the two extremely rare interferences, it has been a reliable alternative to current methods that could easily be placed in production in a small laboratory.

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Is Low Serum Bilirubin an Independent Risk Factor for Coronary Artery Disease in Men But Not in Women? Georg Endler,† Ahmad Hannu,† Raute Sundner-Plassmann,† Markus Exner,† Thomas Vukovich,† Christine Mannhalter,† Johann Wojta,‡ Kurt Huber,‡ and Oswald Wagner†† (1 Clinical Institute of Medical and Chemical Laboratory Diagnostics and 2 Department of Internal Medicine II, Division of Cardiology, University of Vienna Medical School, Währinger Gürtel 18-20, A-1090 Vienna, Austria; * these authors contributed equally to this work; † author for correspondence: fax 43-1-40400-5389, e-mail Oswald Wagner@univie.ac.at)

For many years, the bile pigment bilirubin was considered to be only a toxic waste product formed during heme catabolism. Recent evidence, however, suggests that bilirubin acts as a potent physiologic antioxidant that may provide important protection against arteriosclerosis, coronary artery disease (CAD), and inflammation (1–3). The antioxidant capacity of bilirubin and its potent ability to scavenge peroxyl radicals have led to the concept that mildly increased circulatory bilirubin may have a physiologic function to protect against disease processes that involve oxygen and peroxyl radicals (4). Indeed, inverse correlations between the presence of CAD and total bilirubin concentrations in the circulation were reported recently in several independent studies (5, 6). Additionally, plasma bilirubin correlates inversely with several established risk factors for CAD, including smoking, increased LDL-cholesterol, diabetes, and obesity, but is directly proportional to the protective factor HDL-cholesterol (5, 7). The effect of bilirubin on the risk of cardiovascular disease is apparent in men (8) but is less clear in women (6, 9, 10). In the present study, we therefore examined the influence of gender on total bilirubin concentrations.

All patients referred to the Department of Cardiology, University of Vienna, between August 1999 and September 2001 for whom clinical data were available were included in our study. Patients were divided in a CAD and a non-CAD group. The CAD group consisted of 544 patients (157 females and 387 males) with clinically relevant CAD. Clinically relevant CAD was defined as an exercise-induced ischemic ST-segment depression >0.1 mV (12%) (11) and/or a history of myocardial infarction (53%) or coronary intervention [coronary artery bypass (8%) or percutaneous transluminal coronary angioplasty (27%)]. In the non-CAD group (359 patients; 186 females and 173 males), the presence of CAD was excluded by objective tests that indicate the absence of clinically relevant coronary ischemia (exercise testing and/or thallium-persantin scintigraphy). These patients (non-CAD group, controls) had various diseases, including nonischemic chest pain (23%), valvular disease (13%), nonspecific cardiomyopathy (18%), and nonischemic arrhythmias (38%), and served as controls.

All patients were questioned for established cardiovascular risk factors, including diabetes, smoking (>20 cigarettes/day for more than 5 years), hypertension (systolic blood pressure >140 mmHg or diastolic blood pressure >80 mmHg at repeated measurements or a known history of hypertension and treatment with antihypertensive drugs), body mass index (BMI), and family history of cardiovascular disease. Diabetes mellitus was considered present in patients with a known history of diabetes and in patients with a fasting glucose >7 mmol/L (126 mg/dL) according to American Diabetes Association criteria (12). All blood samples were taken at the time of admission between 0800 and 1000 after an overnight fast. The study was approved by the local ethics committee, and all individuals participating in the study gave informed consent.

We measured serum bilirubin by a diazo method with a detergent to accelerate the azo-coupling and to prevent the precipitation of protein (13). The test was run on a Hitachi 747 (Roche) with a measurement range of 1.0–