Concurrent Measurement of Flecainide Acetate and Propranolol by Normal-Phase High-Performance Liquid Chromatography

Cheryl L. Rongnerud and Ching-Nan Ou

This simple, isocratic, normal-phase liquid-chromatographic method concurrently measures flecainide acetate and propranolol in 100 μL of serum within 8 min. The chromatographic system consists of a Waters "Resolve" column packed with 5-μm silica spheres and a mobile phase of ammonium sulfate (10 mmol/L, pH 6.8) methanol (22/78 by vol), pumped at 0.9 mL/min and monitored by a fluorometer (excitation at 225 nm and emission at 340 nm). After 100 μL of serum is mixed with 200 μL of the internal standard solution [N-(2-piperidylmethyl)-2,3-bis(2,2,2-trifluoroethoxy)-benzamide HCl, 2500 μg/L] and 200 μL of 0.2 mol/L sodium carbonate, the sample is extracted into butanol/hexane (20/80 by vol). The organic layer is separated and evaporated, and the residue is redissolved in 200 μL of methanol; 50 μL of this is injected onto the column. Relative recovery was 100% over the assay range of 25–2000 μg/L for flecainide and 10–2000 μg/L for propranolol. Within-run CVs were <2% for flecainide and <5% for propranolol; day-to-day CVs ranged from 5.0% to 6.5% for flecainide and from 3% to 12% for propranolol.

Additional Keyphrases: antiarrhythmic drug assay . economics of laboratory operation

Flecainide acetate, 2,5-bis(2,2,2-trifluoroethoxy)-N-(2-piperidylmethyl)benzamide acetate, is a relatively new antiarrhythmic agent used to suppress ventricular arrhythmias. Supplied as "Tamboor" by 3M Riker, St. Paul, MN 55144, the drug is clinically effective on oral administration. It has a relatively long half-life, ranging from 12 to 27 h (mean ~20 h) (1). Its optimal effect is recognized when its "trough" concentration (i.e., the concentration measured just before a next dose) is maintained at 200–1000 μg/L (2, 3). On the basis of clinical experience, Nappi and Anderson (1) suggested a therapeutic range of ~400–1000 μg/L for patients without advanced renal failure or congestive heart failure. Toxic effects produced by concentrations of the drug in plasma exceeding this include new or worsened congestive heart failure, exacerbated arrhythmias, and slowed cardiac conduction, as well as dizziness and visual disturbances (1, 2).

During the initial investigational studies in the United States, concentrations of flecainide in plasma were measured by gas–liquid chromatography with electron-capture detection (1). Since then, various HPLC procedures have been developed (4–6). Unfortunately, these lack the sensitivity to detect low concentrations of flecainide unless larger samples are analyzed.

Propranolol is a nonselective beta blocker used in the treatment of hypertension, arrhythmias, angina pectoris, and myocardial infarction (7). Although there is no significant correlation between the concentration of this drug in plasma and the clinical response, propranolol quantification is ordered by physicians to see if a patient is complying with the prescription. The HPLC method described by Ebnet et al. (8) is relatively simple and reliable, but—being subject to interference from quinidine—it must be used with a less-accurate external standard method to calculate the results when quinidine is present.

We describe here a simple, sensitive HPLC procedure for the concurrent measurement of propranolol and flecainide acetate. With a slight change in mobile phase, the system can also be used to quantify amiodarone and its metabolite (9). The ability to quantify more than one drug concurrently and to combine methods with similar operating conditions into one system enables maximum utilization of expensive equipment, minimizes operation cost, and expedites results.

Materials and Methods

Apparatus. We used a Model 5060 ternary-gradient liquid chromatograph interfaced with a Model DS650 Data System and a Model 9090 autosampler (all from Varian Instruments, Palo Alto, CA 94303). The system also included a Kratos Spectroflow 980 fluorometer (Applied Biosystems, Ramsey, NJ 07446) with a 340-nm emission filter, and a 3.9 × 150 mm "Resolve" column packed with 5-μm silica spheres (Waters Associates, Milford, MA 01757). Polypropylene tubes (13 × 100 mm), obtained from American Scientific Products, McGaw Park, IL, and an "N-EVAP" analytical evaporator (Organamation Associates Inc., South Berlin, CA 01549) were used in the sample preparation.

Reagents. The extraction buffer was 0.2 mol/L sodium carbonate, prepared by dissolving 21.2 g of Na₂CO₃ in 1 L of distilled water. The extraction solvent was butanol/hexane (20/80 by vol), both "HPLC" grade (Burdick and Jackson Laboratories Inc., Muskegon, MI 49442). Ammonium sulfate buffer (10 mmol/L) was prepared by dissolving 1.32 g of (NH₄)₂SO₄ in 1 L of doubly distilled water, then filtering it through a 0.45-μm (pore size) Millipore filter and adjusting the pH to 6.8. The mobile phase consisted of this ammonium sulfate buffer and HPLC-grade methanol (22/78 by vol) and was degassed with helium for 5 min before use.

Standards. Flecainide acetate and the internal standard [N-(2-piperidylmethyl)-2,3-bis(2,2,2-trifluoroethoxy)-benzamide HCl] were obtained from 3M Riker. Propranolol HCl was obtained from Sigma Chemical Co., St. Louis, MO 63178.

The concentration of the aqueous stock solution for the internal standard was 1 g/L. The 2500 μg/L working solution was prepared by diluting 2.5 mL of the stock solution to 1 L with water. A 1 g/L aqueous stock solution of flecainide acetate was prepared in a flask pre-seasoned as recommended by S. F. Chang, 3M Riker (personal communication, March 21, 1986): after diluting the stock solution in a volumetric flask and letting it set overnight at room temperature, one discards the original solution and prepares a

Department of Pathology, Texas Children's Hospital and Baylor College of Medicine, Houston, TX 77030.

Received November 28, 1988; accepted February 16, 1989.
new one in the same flask. Working standards for flecainide acetate were also made up in pre-seasoned glassware; concentrations of 100, 600, and 1000 μg/mL were prepared by adding 100, 600, or 1000 μL of flecainide acetate stock solution to a 1-L volumetric flask and diluting to volume with doubly distilled water.

A 1 g/L stock solution of propranolol was prepared by adding 114 mg of propranolol hydrochloride to a 100-mL volumetric flask and diluting to volume with HPLC-grade methanol. Working standards containing propranolol concentrations of 50 and 100 μg/mL were prepared by adding 50 or 100 μL of stock solution to a 1-L flask and diluting to volume with water.

The working standards may also be combined such that standard no. 1 contains flecainide, 100 μg/L; no. 2 contains propranolol, 50 μg/L, and flecainide, 600 μg/L; and no. 3 contains propranolol, 100 μg/L, and flecainide, 1000 μg/L. Pooled, drug-free blood-bank plasma may also be used instead of the water if desired.

**Procedure.** To a 13 × 100 mm polypropylene test tube, add 100 μL of standard, control, or patient's serum; 200 μL of the internal standard working solution; 200 μL of 0.2 mol/L sodium carbonate solution; and 2.0 mL of the butanol/hexane extraction solvent. Vortex-mix for 30 s and centrifuge for 5 min at 1100×g. Transfer the organic (upper) layer to a second polypropylene tube and evaporate to dryness. Dissolve the residue in 200 μL of methanol and inject 50 μL of the solution onto the column. Elute with the mobile phase at the rate of 0.9 mL/min. Monitor the fluorescence of the column effluent by using an excitation wavelength of 225 nm and an emission wavelength of 340 nm.

**Analytical variables.** For the analytical-recovery studies we added both drugs to water and to drug-free plasma to produce concentrations of flecainide at 100, 500, and 1000 μg/L and propranolol at 50 and 100 μg/L and extracted these samples as described above. We also prepared corresponding dilutions of each drug in methanol, but at half the concentrations just listed, to allow for the dilution factor of the reconstitution step. All reconstituted extracted and unextracted samples were injected into the column in triplicate. We made manual injections into a 50-μL sampling loop, to ensure consistent injection volumes. To determine absolute recovery, we compared the peak areas of the extracted samples with those of the unextracted methanol dilutions. To determine analytical recovery, we first calculated the ratio of each drug peak to that of the internal standard. After determining these ratios for the serum-based samples, we then compared them with the corresponding ratios for the aqueous samples.

As the comparison method we used the Abbott fluorescence polarization immunosay protocol for flecainide analysis with the TDx (Abbott Laboratories, North Chicago, IL 60064) as described in the manufacturer's manual.

**Results and Discussion**

Propranolol, flecainide acetate, and the internal standard are well resolved within an 8-min run time as shown in Figures 1 and 2. Retention times are 5.4, 6.3, and 7.1 min for propranolol, flecainide acetate, and internal standard, respectively.

In the liquid-extraction procedure, we saw no differences in analytical recovery between the aqueous and the serum-based standards. The absolute recovery of either drug from serum, as compared with an appropriately diluted unretracted methanol solution, was 85%. When we evaluated a solid-phase extraction procedure described by Chang et al. (5), the flecainide samples prepared by either method showed identical recovery. Propranolol, however, showed significantly lower recovery in the solid-phase extraction procedure. Chromatograms produced by samples extracted by both methods showed very little difference other than a slightly larger injection peak after the liquid-extraction procedure. We preferred the liquid-extraction procedure because of its improved recovery, simpler technique, and lesser cost.
Linearity of their standard curves to 2000 μg/L was established for both drugs. Detection limits were 25 μg/L for flecainide and 10 μg/L for propranolol. Within-run CVs (n = 10) were 1.6% for flecainide acetate at a concentration of 577 μg/L and 4.2% for propranolol at a concentration of 95 μg/L. Using the TDx Flecainide Controls (Abbott no. 9799-10) for flecainide, and Lyphocheck Trilevel Therapeutic Drug Monitoring Controls (Bio-Rad Labs., ECS Division, Anaheim, CA 92806) for propranolol, we calculated day-to-day precision data for each drug over a six-month period. The CVs for flecainide were 6.5% at x = 236 μg/L, 5.8% at x = 591 μg/L, and 5.0% at x = 1198 μg/L. Those for propranolol were 12.0% at x = 41 μg/L, 7.8% at x = 90 μg/L, and 3.3% at x = 214 μg/L. The means obtained for flecainide by HPLC for each Abbott control were very close to the stated target values of 300, 600, and 1200 μg/L. The TDx results, although remaining within the stated range, always exceeded the mean.

Figure 3 shows a chromatogram for a Lyphocheck TDM Level III control sample. The large peaks seen after the internal standard represent quinidine, dihydroquinidine, N-acetylprocainamide, and procainamide. None of the 33 drugs present at high concentrations in this control material interferes with the assay. The small unidentified peak in the flecainide region has a retention time that is 0.1 min longer than flecainide. Should it be mistakenly identified as flecainide, it would be reported at a concentration of about 15 μg/L, which is below the stated detection limit of the assay and not clinically significant. Other drugs tested and shown not to interfere with the assay include enexazide, ibuprofen, naproxen, indomethacin, tocainide, amiodarone, N-des-ethylamiodarone, and verapamil.

To determine method correlation between HPLC and TDx results for flecainide, we began with a calibration crossover study. In-house-prepared, plasma-based calibrators were compared with commercial TDx flecainide calibrators. TDx calibrators assayed by the HPLC method showed the relationship: y (measured) = 0.90x (prepared) + 9 μg/L (R² = 0.999, n = 6). When the HPLC calibrators (25, 100, 600, and 1000 μg/L) were assayed by the TDx, the correlation was: y (measured) = 1.1x (prepared) (R² = 0.999, n = 4).

Figure 4 illustrates the correlation between flecainide concentrations in patients’ serum samples as assayed by the two methods. These actual measured results, with each assay being standardized with its own set of calibrators, show that the HPLC results are consistently lower than those by the TDx, as demonstrated by the slope of the correlation. Although this indicates an inaccuracy in one or both of the methods, the correlation between the two is consistent (R² = 0.980). After we corrected for the difference in the two sets of calibrators (TDx result × 0.9 plus 9 μg/L), the correlation between TDx (y) and HPLC (x) results was as follows: y = 0.93x + 32 μg/L (R² = 0.980). Similar correlation results have also been observed elsewhere (SF Chang, 3M Riker, personal communication). We have also noted that samples with flecainide concentrations ≤200 μg/L tended to give higher results by HPLC than by TDx, whereas the opposite was true for concentrations exceeding 200 μg/L. Correlation of patients’ data for only those samples with flecainide concentrations within the therapeutic range (200 – 1000 μg/L) showed the relationship: y (HPLC) = 0.95x (TDx) + 19 μg/L (R² = 0.987, n = 29).

Straka et al. (10) also correlated TDx (y) and HPLC (x) results for flecainide in patients’ samples. Their HPLC procedure included the solid-phase extraction described by Chang et al. (5) and a Waters μ-Bondapak phenyl column. Their equation for the correlation was y = 0.97x + 13 μg/L (r = 0.979, n = 32). They did not state, however, whether any crossover studies were performed with the two sets of calibrators and controls used. Commercial (Abbott) calibrators and controls were used for the TDx assay and flecainide-supplemented serum calibrators and controls for the HPLC procedure.

Abbott Laboratories cited three additional studies (3) in which patients’ samples were analyzed for flecainide by both the TDx (y) and by the HPLC (x) procedure of Chang et al. (5). Using the TDx as the test method, they obtained the following three correlations: Site 1, y = 1.03x + 5 μg/L (r =
immunoglobulins

There does appear to be some variability in the calibrators from site to site, as shown by the differences in slopes. Some of this variation may be attributable to the use of seasoned vs unseasoned glassware in preparing calibrators. We have observed that dilutions prepared in unseasoned glassware produce much more erratic and slightly lower results than identical dilutions prepared at the same time in seasoned flasks. The flecainide assay appears to be more affected by this than the propranolol assay, but this variability is not sufficient to account completely for the difference between the two sets of calibrators. In any case we suggest the use of the pre-seasoning technique, because we find that produces very consistent and (we believe) accurate calibrator concentrations.

The column used in this method has proven to be very durable. We routinely can make more than 1000 injections before the column starts to deteriorate. We use 2 L of mobile phase, recycling it during the analysis. After about a month (or about 200–300 injections) the chromatograms start to show a trough immediately after the internal standard. Usually we prepare fresh mobile phase at this time, because the trough makes it more difficult for the data system to integrate peak area accurately. Once or twice during the useful life of a column, we change the inlet column filter to correct for a slight increase of pressure in the system. We also use this same column and pump for amiodarone analysis by simply equilibrating the column for 15 min in an amiodarone mobile phase that is very similar to that of the flecainide assay (9).

In conclusion: this assay permits accurate, expedient quantification of both flecainide acetate and propranolol in serum. Its high sensitivity requires only a 100-μL sample.


Application of a Silver-Binding Assay to the Determination of Protein in Cerebrospinal Fluid

Gerald Krystal,1,2 Vivian Lam,1 and William E. Schreiber1,3

We evaluated a silver-binding assay for use in measuring total protein in cerebrospinal fluid. The advantage of this procedure over other methods is that, because of its sensitivity, it requires only a 0.5-μL sample. The procedure, which takes approximately 40 min to complete, involves dilution of 0.5-μL samples to 1 mL with distilled water containing sodium dodecyl sulfate, followed by addition of glutaraldehyde and an ammonical silver solution. After color development for 30 min, the reaction is terminated with sodium thiosulfate and the absorbance is measured at 420 nm. This assay displayed within-run and day-to-day precision (CV) of 3.1% to 13% over the range of 210 to 1370 mg/L. It showed substantially less protein-to-protein variation than the Coo-massie Blue dye-binding procedure when tested with albumin, globulin, and transferrin. It also yielded an accurate estimation of hemoglobin. Moreover, preliminary studies suggested that it was capable of quantifying immunoglobulin light chains and glycoproteins. In a study of 54 human cerebrospinal fluid samples, results of the silver-binding assay corresponded more closely with those obtained with a rate biuret assay (intraclass correlation coefficient = 0.91) than did either the dye-binding or classical Lowry methods.

Additional Keyphrases: hemoglobin • immunoglobulins • glycoproteins • pediatric chemistry • reference values by various procedures

Total protein determinations, an integral part of cerebrospinal fluid (CSF) analysis, are most commonly performed by turbidimetric, dye-binding, or biuret assays. In most

1 The Terry Fox Laboratory, B.C. Cancer Research Centre, 601 West 10th Ave., Vancouver, B.C., Canada V5Z 1L3.
2 Department of Pathology, University of British Columbia, Vancouver, B.C. V6T 2B5.
3 Division of Clinical Chemistry, Vancouver General Hospital, 855 West 12th Ave., Vancouver, B.C. V5Z 1M9.

Received October 12, 1988; accepted February 17, 1989.

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4 Nonstandard abbreviations: CSF, cerebrospinal fluid; CBB, Coo-massie Brilliant Blue G-250 (a registered trademark of Imperial Chemical Industries, Ltd.); and SDS, sodium dodecyl sulfate.