Automated Fluorimmunoassay of Theophylline and Valproic Acid by Flow-Injection Analysis with Use of HPLC Instruments

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For automated determination of theophylline and valproic acid by use of Ames' fluorimmunoassays we used a high-pressure liquid-chromatographic system consisting of a pump, a robotic unit (Gilson 231/401, to prepare and inject the samples into the flowing carrier), and a fluorometer with a 10-μL flow cell. Results correlated well with those of conventional liquid- and gas-chromatography (r > 0.96). The between-run CV is about 5%. In comparison with the manual method, the volume of reagents (and thus the cost per analysis) was decreased by eightfold.

The last decade has seen the introduction of several drug immunoassays. Currently, automated instruments allow measurements of absorbance, fluorescence, fluorescence polarization, or luminescence, and these methods are now being used for routine drug monitoring. For example, the enzyme immunofluorassay developed by Burd et al. (1) and marketed by Ames was adapted by Li et al. (2) to an automated fluorometer.

Since its introduction in 1975 by Ruzicka and Hansen (3), flow-injection analysis is a rapidly growing technique, because any type of detector can be used as the sample injected into a continuous-flow carrier is transported into the detector.

Here we describe results obtained for theophylline and valproic acid determination with the Ames fluoroenzymoimmunoassay performed with standard high-pressure liquid chromatography (HPLC) instruments, used without a column in a flow-injection-analysis mode.

Materials and Methods

The reagents used are those for the Ames therapeutic drug assay (Ames Div. of Miles Labs., Elkhart, IN). Each kit includes a 5-mL bottle of antibody–enzyme reagent (antisem to drug and β-galactosidase), a 5-mL bottle of fluorogenic drug reagent (β-galactosyl umbellif erone–drug conjugate), four calibrators in human serum, and a "bicine" buffer. [Bicine is N,N-bis(2-hydroxyethyl)glycine.]

The drugs we tested were theophylline and valproic acid.

The apparatus consisted of a HPLC pump, a robotic unit, a spectrofluorometer, and a data system. The pump, an LKB Model 2150, was set at a flow rate of 1.75 mL of water per minute. The robotic unit was the Gilson Model 231/401 with a 500-μL syringe, used for the preparation of the samples and their injection into the flowing carrier.Disposable 1-mL plastic tubes, empty or containing the two reagents, calibrators, and samples to be analyzed, were placed in an aluminum rack, thermostated at 30 °C by water circulation. The Gilson unit was programmed to prepare the blood plasma calibrators and samples in the same way as the manual procedure given by Ames, except that the volumes used were eightfold less. Preparation for the automated procedure consists of:

1) two successive dilutions of plasma: first, 6 μL + 300 μL of buffer, then 6 μL + 60 μL of buffer,
2) additions to the diluted plasma of 6 μL of fluorogenic drug reagent + 60 μL of buffer, then 6 μL of antibody enzyme reagent + 60 μL of buffer.

After a 16-min incubation at 30 °C, the Gilson unit injects 65 μL of each prepared sample into the flowing carrier via its loop filler port. A second similar injection was made immediately after the first and the two measurements were averaged.

The detector we used was a Kontron spectrofluorometer SFM 25 with 10-μL flow cell, set at 405 nm for excitation and 450 nm for emission, at a response time of 0.5 s, and at a photomultiplier voltage of 500 to 525 V.

The signals from the spectrofluorometer were analyzed with an Apple II E microcomputer, with use of an ADALAB interface card (Interactive Microware) and a homemade program that gives the drug concentration directly from the calibration curve.

To assess the validity of the described method, samples from treated patients were analyzed both by the present method and by techniques routinely used in therapeutic drug monitoring. Theophylline was determined by an HPLC method on a reversed-phase column (Spherisorb C6) after an extraction adapted from Peat et al. (4). Valproic acid was monitored by a gas-chromatographic method on a 5% FFAP column with caprylic acid as internal standard after an extraction with carbon tetrachloride as described by Dijkstra and Vervloet (5).

Least-squares linear regression analysis was performed according to Davis et al. (6) and the 95% joint confidence intervals of the slope and intercept were determined. Prediction intervals for a single observation (7) were also calculated.

Results

Figure 1 shows typical responses from the blank, the theophylline calibrators, and the patients' samples. There is no noise or baseline drift, and the two responses for the same sample are similar. Figure 2 shows the mean calibration curve obtained from eight determinations.

The reproducibility of the method for within-run assays and between-run assays were respectively 4.5% and 6.4% for theophylline at the concentration of 13.8 mg/L and 3.7% and 6.6% for valproic acid at the concentration of 55 mg/L. Because the needle can be rinsed every time, there is no cross contamination between samples.

Least-squares linear regression analysis (Figures 3 and 4) indicated good correlation (r > 0.96) between results by the present method and those by conventional HPLC or gas chromatography. In each case, the values of the slope and intercept, expressed as mean plus or minus the joint confidence interval, were close to values of 1 and 0, showing a good identity between the two methods.

Thus, such determination of theophylline and valproic acid...
Fig. 1. Responses for the blank (1–2), four calibrators (3–6), and samples (7–20), each measured twice (theophylline assay).

Fig. 2. Calibration curve for theophylline as calculated by the computer. Bars indicate ±2 SD (n = 8).

Fig. 3. Regression [equation: \( y = (a \pm JCI) \cdot x + (b \pm JCI) \)] of theophylline concentrations as determined by the two methods. JCI: 95% joint confidence interval. Solid line: Ames = \( (1.02 \pm 0.03) \cdot \text{HPLC} – (0.06 \pm 0.04); n = 138; r = 0.98; s_w = 11 \). Dotted line: prediction interval for an estimated \( y \).

Fig. 4. Regression [equation: \( y = (a \pm JCI) \cdot x + (b \pm JCI) \)] of valproic acid concentrations as determined by the two methods. JCI: 95% joint confidence interval. Solid line: Ames = \( (1.01 \pm 0.03) \cdot \text{GLC} – (0.56 \pm 1.60); n = 123; r = 0.96; s_w = 6.6 \). Dotted lines: prediction interval for an estimated \( y \).

Valproic acid can be automated satisfactorily by flow injection analysis after the sample is prepared by a robotic unit. The sensitivity, less than 1 mg/L for theophylline and valproic acid, suffices for their therapeutic monitoring. Interferences, not studied here, are expected to be the same as with the manual procedure, because the same ratios of sample and reagents were used in the two procedures.

Discussion

The heart of the system described is the Gilson Model 231/401, which is easily programmed to perform almost any homogeneous liquid preparation with addition of one or several reagents and injection into the flowing carrier. It requires only microliter aliquots, which decreases the cost per analysis.

Because all the units of this system are dedicated to HPLC analysis, connection of a column to the injection valve and the detector quickly provides the complete standard HPLC instrument.

References