Fluorescence Polarization Immunoassay. III. An Automated System for Therapeutic Drug Determination

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A fully automated system for performing fluorescence polarization immunoassay has been developed. Reagents for each assay are contained in coded reagent packs, and no reagent reconstitution is required. A common buffer is used for all assays, minimizing changeover and set-up times for each assay. A single sample may be assayed in 5 min, or 20 samples in 10 min. A single-tube blank subtraction for each sample results in highly precise polarization values and obviates sample interferences. We have used this method for assays of gentamicin, theophylline, phenytoin, and phenobarbital. CVs are 1–4%, and the results correlate well with those by other methods. Because of the instrument design and the stability of the reagents, daily calibration is not required; samples may therefore be run immediately upon receipt or batched as desired.

Additional Keyphrases: *gentamicin* · *theophylline* · *phenytoin* · *phenobarbital*

The principles of fluorescence polarization were first developed in the 1920s by Perrin (1). Three decades later the technique was applied to biological systems by Weber (2), and its application to the antigen–antibody reaction was first described in 1961 by Dandliker and Feigen (3). The principles and practice of fluorescence polarization and its application to biological systems have been the subject of several review articles (2, 4, 5).

Fluorescence polarization immunoassay (FPIA)[1] makes use of competitive-binding assay principles, measuring tracer binding directly, without the need for a separation procedure. The specificity of an immunoassay is thereby combined with the speed and convenience of a homogeneous method, providing a precise and reliable procedure for determining the concentrations of biologically interesting substances in serum and plasma. FPIA has been applied to the measurement of such compounds as insulin (6), cortisol (7), gentamicin (8, 9), and phenytoin (10, 11).

Despite the advantages of FPIA and its great potential as an assay technique, the method has seen little clinical utility, primarily because of the lack of simple, low-cost, high-performance instrumentation. Several different instrumental designs have been described (6, 12–14), some of which are available commercially; in addition, most manufacturers of research spectrofluorimeters offer polarization accessories. None of these instruments, however, is suitable for the routine clinical laboratory. We have recently described a simple, rapid, and precise instrument (15) and companion reagent systems

for determination of the aminoglycoside antibiotics gentamicin, tobramycin, and amikacin; the anticonvulsants phenytoin and phenobarbital; and the anti-asthmatic drug, theophylline (9, 11, 16). Here we describe the automation of these assays, which yields results superior to the manual methodology in terms of precision, speed, and ease of use.

Materials and Methods

Apparatus

We have constructed a totally automated, bench-top, fluorescence polarization analyzer, in which all processing is carried out in a light-tight, temperature-controlled environment. The major components, shown in Figure 1, are a dual-syringe pump, liquid and air heaters, a pipettor boom, a polarization fluorimeter (15), microprocessor-based electronics, an alphanumeric printer, and a control panel. A 20-position carousel carries sample cups and cuvettes; tracer, antibody, and pretreatment reagents are contained in a three-chamber pack. Each pack has a bar-coded label that identifies the assay to the instrument. Buffer common to all assays is always on-line to the pump and is preheated immediately before delivery. The specimen (>50 μL) is placed in the sample side of the dual-chamber sample cup. A sample cup and cuvette are required for each standard, control, or patient's specimen. Reagent packs for the assay are stored at 4–8 °C, but can be loaded into the instrument without equilibration to room temperature. After loading the carousel and reagent pack, start the processing by closing the door and pressing the run button.

Set-up is automatic. The bar-code on the reagent pack is read and processing parameters are retrieved from non-volatile memories. The number of samples to be processed is determined by an optical sensor, and all reagent volumes are checked with a liquid-level sensor on the tip of the probe. The cuvettes are warmed to 35 °C by an air heater controlled through an infrared sensor mounted in the optics module. This typically requires less than 30 s. Temperature is maintained at 35 ± 0.5 °C with forced air heat.

On the first revolution, samples are diluted with buffer in the dilution well of the sample cup. Typically, 20 μL of sample is mixed with 700 μL of buffer. The carousel then moves to the next sample position. On the second revolution, 10 to 25 μL (as required) of diluted sample and 25 μL of pretreatment solution are added to the cuvette along with buffer to a total volume of 975 μL. A blank reading is then made when the cuvette reaches the fluorimeter. For partial batches, the carousel is advanced with minimum delay when samples are not present. On the third revolution of the carousel, 25 μL of tracer, 25 μL of antibody, and a volume of diluted sample equal to that used in the second revolution are added with buffer to bring the total volume to 1.95 mL. After a 3-min incubation, the final polarization measurement is made on a fourth revolution. A blank-corrected polarization value is calculated (15) and either a standard curve is computed or the concentrations of specimens are determined by reference to a stored curve. A weighted log-logit-type curve fit is used. A
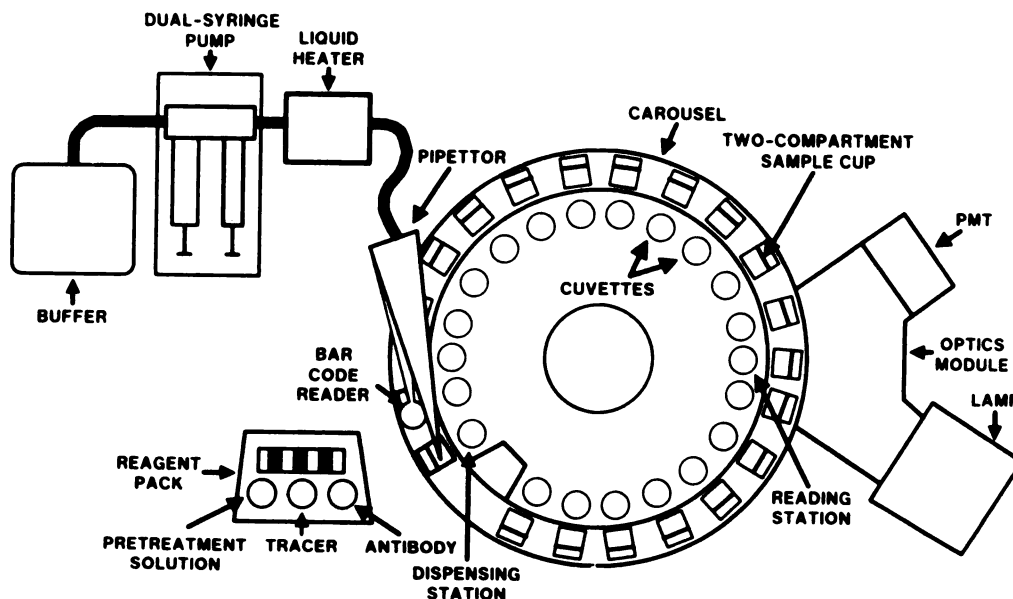
Fig. 1. The major components of the totally automated, bench-top, fluorescence polarization analyzer

PMT, photomultiplier tube

hard-copy report is made with the 40-column printer. A full batch of 20 samples requires less than 10 min between loading samples and printout of the final results. A single determination is completed in less than 5 min.

With the control panel, one can adjust the assays to be run and reported, changing replications, dilutions, and standard concentrations. Also, limits on reagent performance are stored for each assay, to check the integrity of samples and reagents. Numerous self-diagnostics are also provided for trouble-shooting problems and verifying system performance.

Reagents

The buffers and active components are identical to those previously described for gentamicin, theophylline, phenobarbital, and phenytoin (9, 11, 16); however, the concentrations of tracers and antisera were changed to allow optimum performance at 35 °C. In addition, the tracers and pretreatment reagents (for releasing bound drug from serum proteins) have been separated to facilitate the automated blank-subtraction procedure. Pure drugs were obtained from the U.S. Pharmacopoeial Convention, Inc., Rockville, MD 20852, and were used according to the supplier's instructions.

Pooled, drug-free normal human serum was used throughout. The standards and controls were prepared by dilution from a concentrated stock solution, the concentration of which was verified by "high-performance" liquid chromatography (HPLC) (17-19). Surplus clinical specimens were obtained from hospital and reference laboratories, and were shipped and stored frozen.

Reference assay kits were obtained from commercial sources and used according to the manufacturer's instructions. RIAs were from Diagnostic Products, Los Angeles, CA 90064; New England Nuclear, North Billerica, MA 01862; and Clinical Assays, Cambridge, MA 02139. Homogeneous enzyme immunoassay (EMIT™) was from Syva Co., Palo Alto, CA 94304; and a substrate-labeled fluorescent immunoassay (SLFIA) was from Ames Div., Miles Laboratories, Elkhart, IN 46515. HPLC was performed according to published procedures (17-19). Correlations between methods were determined by linear regression analysis.

Results

Standard curves. Typical standard curves for gentamicin,

phenytoin, phenobarbital, and theophylline are shown in Figure 2. Stored at 2–8 °C, the reagents for gentamicin yield superimposable standard curves for at least one year in the manual system (Figure 3). The reagents are also stable for at least one week at 37 °C and 45 °C (Table 1). All FPIA reagents must show similar stability to heat stress to be acceptable. Because the automated assays are run under strict time and temperature control there is, therefore, no requirement to include standards in each batch of assays. The standard curve may be stored in the instrument and has been shown to be valid for at least 10 days (Table 2).

Precision. The reproducibility of the automated system was determined by supplementing normal human serum samples with three clinically significant concentrations of drug and making five assays of five replicates each. The concentration of each replicate was determined from a single standard curve derived from a previous run. Reproducibility of the manual

Table 1. Stability of the Gentamicin FPIA Reagents at Various Temperatures

Temp., °C	Assay span,[a] mP, on day					
	0	1	2	3	4	7
2–8	132	136	131	132	129	131
37		136	131	130	130	133
45		132	127	134	127	133

[a] Polarization of the 0 mg/L standard minus polarization of the 8 mg/L standard (manual system).

Table 2. Phenobarbital Standard Curve by Automated FPIA

Concn, mg/L	Fluorescence polarization, mP[a]			
	Day 0		Day 10	
0	295	293	295	295
5	265	265	264	265
10	240	240	239	239
20	205	206	207	204
40	169	169	169	168
80	131	133	131	131

[a] Duplicate determinations.

POLARIZATION (mP)

GENTAMICIN CONCENTRATION (mg/L)

POLARIZATION (mP)

PHENOBARBITAL CONCENTRATION (mg/L)

POLARIZATION (mP)

THEOPHYLLINE CONCENTRATION (mg/L)

POLARIZATION (mP)

PHENYTOIN CONCENTRATION (mg/L)
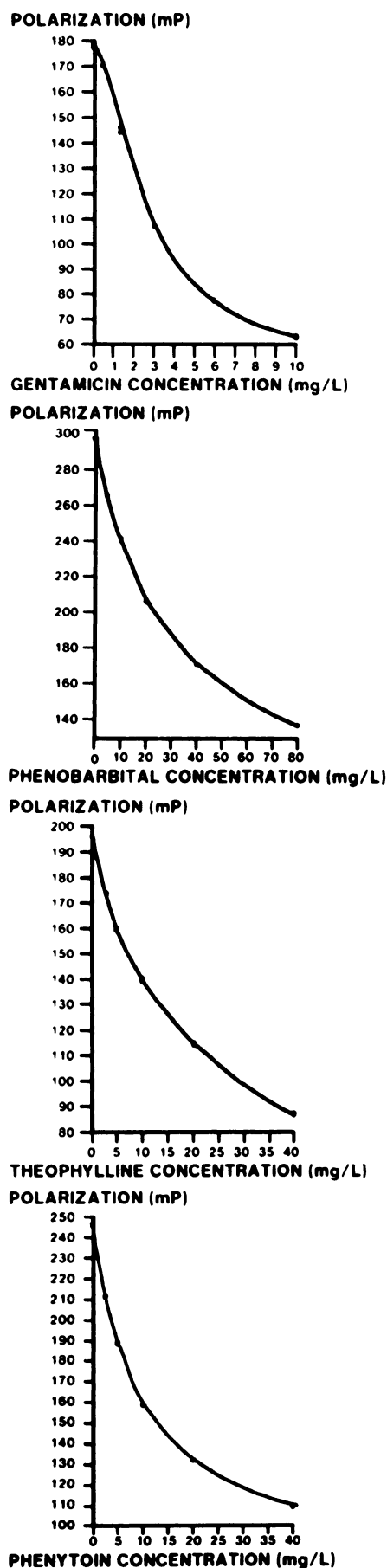
Fig. 2. Typical standard curves for gentamicin, theophylline, phenobarbital, and phenytoin

POLARIZATION (mP)
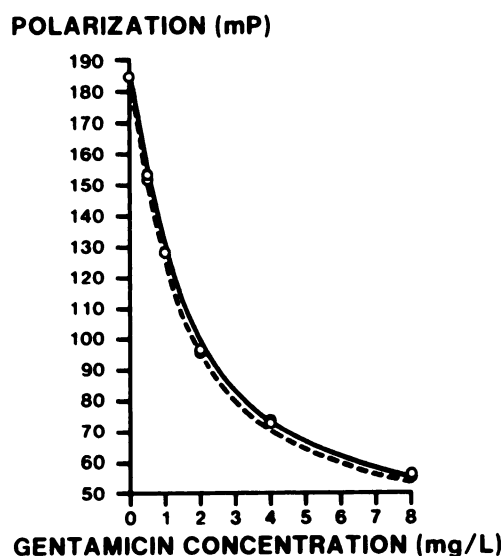

GENTAMICIN CONCENTRATION (mg/L)

Fig. 3. Stability of gentamicin reagents stored at 2–8 °C, as shown by superimposable standard curves
—O—, Day 389; — — —, Day 0

system was determined by assaying five replicates, twice per day for five days (9, 11, 16). The means, standard deviations (SD), and coefficients of variation (CV) for the automated and manual systems are summarized in Table 3. Between-run precision of the automated system was such that CVs were <4% for every assay at every concentration tested, compared with <8% for the manual system.

Correlation with commercially available assays. The gentamicin results by FPIA were compared with those by three commercially available RIA kits, EMIT, and SLFIA. The phenytoin and phenobarbital results by FPIA were compared with those by EMIT, SLFIA, and HPLC. The theophylline FPIA results were compared with those by EMIT, RIA, and HPLC. Table 4 summarizes the data. The good correlations between the automated FPIA and the commercially available methods indicates that the automated system accurately determines drug concentrations in clinical specimens.

Discussion

We have recently described reagents (9, 11, 16) and instrumentation for the fluorescence polarization immunoassay of gentamicin, tobramycin, amikacin, phenytoin, phenobarbital, and theophylline in human serum and plasma. These assays were performed manually and have been shown in field evaluations to be clinically useful (manuscripts in preparation). The automation of these assays has improved precision (CV), speed, and ease of use. In addition, the automated instrument subtracts a blank for each sample, which is necessary for the occasional highly icteric sample, to avoid underestimation of gentamicin and tobramycin concentrations (9). The blank subtraction is performed by a single-tube procedure. After the predilution cycle one-half of the final volume of the prediluted sample and one-half of the final volume of the buffer are combined with the pretreatment reagent, and the vertical and horizontal fluorescence intensities of the blank are determined. Tracer, antibody, and the second half of the prediluted sample are then added with the rest of the buffer. After a 3-min incubation period, the vertical and horizontal fluorescence intensities are again determined; those of the blank are subtracted and the blank-subtracted polarization is determined for each sample. This technique also subtracts stray and scattered light and ensures fluorescence polarization measurements of the highest accuracy by eliminating cuvette variations and nonlinear dilution errors often seen with serum

Table 3. Precision of the Automated vs the Manual FPIA [a]

Gentamicin	Automated			Manual		
Target value, mg/L	1.0	4.0	8.0	1.0	3.0	7.0
Mean, mg/L	1.03	4.09	7.90	1.12	3.23	7.02
SD, within-run, mg/L	0.027	0.069	0.202	0.059	0.149	0.306
CV, within-run, %	2.63	1.67	2.55	5.32	4.62	4.36
SD, between-run, mg/L	0.035	0.091	0.214	0.077	0.204	0.483
CV, between-run, %	3.44	2.22	2.72	6.94	6.32	6.89
Theophylline						
Target value, mg/L	7.0	12.0	26.0	6.0	11.0	22.6
Mean, mg/L	7.18	12.29	26.39	6.43	11.69	23.45
SD, within-run, mg/L	0.178	0.259	0.359	0.34	0.59	0.86
CV, within-run, %	2.47	2.10	1.35	5.27	5.03	3.68
SD, between-run, mg/L	0.220	0.286	0.402	0.39	0.64	1.34
CV, between-run, %	3.06	2.32	1.52	6.02	5.46	5.71
Phenobarbital						
Target value, mg/L	15.0	30.0	50.0	15.0	30.0	50.0
Mean, mg/L	15.18	29.40	48.67	15.95	30.27	49.88
SD, within-run, mg/L	0.208	0.369	0.741	0.721	0.879	1.64
CV, within-run, %	1.37	1.25	1.523	4.56	2.90	3.30
SD, between-run, mg/L	0.258	0.423	0.813	1.02	1.58	2.60
CV, between-run, %	1.70	1.43	1.67	6.42	5.23	5.21
Phenytoin						
Target value, mg/L	7.5	15.0	30.0	7.50	15.0	30.0
Mean, mg/L	7.49	15.23	30.41	7.50	14.93	29.54
SD, within-run, mg/L	0.17	0.33	0.42	0.308	0.525	1.30
CV, within-run, %	2.31	2.16	1.39	4.11	3.51	4.40
SD, between-run, mg/L	0.18	0.35	0.48	0.47	0.74	1.59
CV, between-run, %	2.46	2.31	1.57	6.31	4.97	5.36

[a] See text for details.

Table 4. Correlation of FPIA (y) with Other Methods (x)

	No. of observations	y-Intercept	Slope	r
Gentamicin				
RIA A	46	0.379	0.686	0.970
RIA B	38	−0.536	1.092	0.979
RIA C	153	−0.756	1.002	0.958
EMIT	78	0.198	0.984	0.944
SLFIA	58	0.330	0.911	0.942
Theophylline				
RIA	38	1.476	1.022	0.935
EMIT	97	0.016	0.939	0.976
HPLC	97	−0.091	0.979	0.984
Phenobarbital				
EMIT	165	1.023	0.956	0.992
SLFIA	179	0.561	0.919	0.985
HPLC	37	2.274	0.986	0.970
Phenytoin				
EMIT	170	−0.241	0.923	0.987
SLFIA	197	0.154	0.971	0.946
HPLC	34•	−0.375	0.932	0.985

fluorescence (unpublished observations). In addition, one can determine substances present at very low concentrations, for example, digoxin, where a larger sample volume and lower tracer concentration are required (unpublished results).

All functions of the instrument are under microprocessor control. Incubation time, temperature, and pipetting are maintained to close tolerances. Together with the great stability of the FPIA reagents, these factors allow reproducible standard curves to be obtained from day to day. Under routine use in the clinical laboratory only one standard curve, which is stored in the computer, need be run per assay kit. This allows stat or batch operation without the need to include a standard curve with each run. Appropriate controls should be included to verify system performance, however.

All assays rely on a common buffer, which is contained within the instrument, and a separate reagent pack, so that change-over from one assay to another is fast and simple.

Finally, the performance of the system has improved significantly over the manual system. A 50% reduction in CVs is routinely observed. In addition, the time required for the assay of 20 samples has been reduced by one-half, and attended time reduced to essentially zero.

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