Stopped-flow Fluorescence Polarization Immunoassay

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Abstract: A general survey of the analytical application of kinetic methodology in fluorescence polarization immunoassay (FPIA) is presented. Stopped-flow mixing technique (SF) allows the initial rate of the immunochemical reaction between the tracer and the antibody to be obtained, which is used as the analytical parameter instead of the equilibrium signal used in conventional FPIA. The instrumentation required is described and the features of the analytical methods proposed are compared with those obtained by conventional FPIA. The usefulness of SF-FPIA for routine screening in clinical, environmental and food analysis is discussed.

Key Words: Kinetic methodology, stopped-flow, T-spectrofluorimeter, therapeutic and abused drugs, pesticides.

INTRODUCTION

Since the first immunoassay involving the use of radioisotopes was reported, a wide variety of alternative immunoassay methods were developed to overcome the shortcomings of radiolabeled reagents. Thus, several homogeneous fluorescence immunoassays were described [1], which also avoided the separation steps required in radioimmunoassay (RIA), but the poor sensitivity and limited applicability of some of them hindered their application to the analysis of real samples. However, the special nature of fluorescence polarization, and its dependence on the size of the molecules involved in the immunological reactions are the main reasons for wide use of fluorescence polarization immunoassays (FPIAs) for the homogeneous determination of haptens. The examples of these haptens are therapeutic and abuse drugs and pesticides [2-4]. Therefore these FPIAs can be seen as a useful alternative to RIA. In fact, of all the fluorescence immunoassays developed to date, FPIA has been one of the most successful.

As for any homogeneous immunoassay, a limitation of FPIA when applied to the analysis of real samples is the relatively high detection limits obtained as a result of the background signal, which is caused partly by scattered light and partly by the fluorescence of the sample matrix. Scattered light can be originated by macromolecules such as proteins and, also, by the fluorescent substance used as the label that has a small Stokes shift. Fluorescein (the main fluorescent reagent used as a label in FPIA) is such a molecule that serves this purpose; although its maximum excitation and emission wavelengths are very close (λ_{ex} 495, λ_{em} 520 nm). An alternative approach to avoid or minimize this effect, is to use the initial rate of the immunochemical reaction between the tracer and the antibody as the analytical parameter, instead of the signal obtained when the reaction reaches or is close to the equilibrium. As the initial rate of the competitive antigen-antibody reaction is usually very fast, kinetic data can be obtained by using stopped-flow mixing technique (SF). This allows the measurements to be performed shortly after mixing the reagents and, in addition, to reduce reactants manipulations and automate the measurement step of the analytical process [5]. Unlike homogeneous enzyme immunoassay, in which kinetic methodology is used to determine the enzyme activity of the free tracer with an additional reagent after the competitive immunochemical reactions have finished, SF-FPIA is applied directly to the tracer-antibody reaction, which develops simultaneously with the analytical reaction. Moreover, because SF has traditionally been used for physicochemical studies, one can conduct fundamental kinetic studies in FPIA.

Although the analytical applications of SF-FPIA have been relatively limited, it has shown its usefulness to improve some features of conventional FPIA for the direct determination of therapeutic (nordiazepam and imipramine) [6] and abused (opiates, d,l-amphetamine, cocaine and cannabinoids) [7,8] drugs in serum and urine samples and pesticides (atrazine and 2,4-dichlorophenoxyacetic acid, 2,4-D) [9,10] in food and environmental samples, as it is discussed below. All these methods were developed using fluorescein derivatives as tracers.

THEORETICAL AND PRACTICAL ASPECTS OF SF-FPIA

FPIA is a competitive-binding assay where the tracer (the antigen conjugate) binding with the antibody is directly measured without the need for a separation step. It relies in the fact that, when a molecule is excited by polarized light, the emitted light will also be polarized if the molecule does not rotate during the time elapsed between excitation and emission. Small fluorescent molecules rotate rapidly and normally exhibit no fluorescent polarization; however, on binding to macromolecules, the rotation motion is slowed down and the fluorescence remains polarized. Thus, the tracer, which competes with the analyte for the antibody, will only exhibit polarized fluorescence when bound to the antibody, and this will be inversely proportional to the analyte concentration.

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The polarized fluorescence is usually measured at equilibrium, even though it can also be measured during the reaction by monitoring the evolution of the tracer-antibody system. As known, the fluorescence polarization (FP) is quantified via the degree of polarization, given by $P = (I_V - I_H)/(I_V + I_H) = [(I_V/I_H) - 1]/[(I_V/I_H) + 1]$, where I_V and I_H are the fluorescence intensities measured with the emission polarizer parallel and perpendicular, respectively, to the excitation polarizer, placed to obtain vertically plane polarized light. Measuring the variation of P with time (dP/dt) allows one to obtain the reaction rate (V_P) of the tracer-antibody complex, which is also inversely proportional to the analyte concentration.

The only requirement for application of kinetic methodology in FPIA is the availability of instrumentation that permits simultaneous measurements of the variation of I_V and I_H with time, to be processed by the computer in order to construct a polarization-time kinetic curve. Although automatic commercial FPIA instruments are available, they are "black-box" systems where the user is fully dependent on the supplier for assay procedures and cannot develop his own methods. However, kinetic FPIA measurements can be obtained by using a T-spectrofluorimeter with two emission channels symmetrically arranged on both sides of the sample compartment and the emission polarizers perpendicular to each other. The instrument is fitted with a SF module which allows the initial rate of the immunochemical system to be obtained. The SF mixing technique is widely used in kinetic and mechanistic studies of fast reactions and, also, is a useful means of accomplishing automation in kinetic analysis by using these type of reactions [5]. It uses two drive syringes that are actuated manually or automatically (by a pneumatic device) to allow two reactant streams (sample and reagents) to be accelerated and rapidly merged into the mixing chamber, after which the flow is suddenly stopped by using a third, stopping syringe, and the analytical signal is monitored. Usually, the mixing chamber is the observation cell and is located inside the cuvette holder of the instrument. The experimental set-up used is shown schematically in figure **1**. The kinetic curve is displayed on the computer screen and the data are processed by appropriate software.

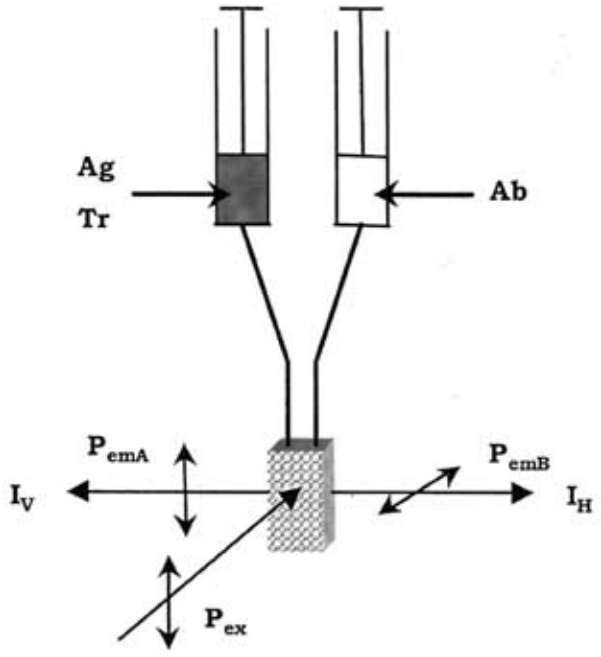

Fig. (1). Scheme of the SF-FPIA experimental set-up. Ag: analyte; Tr: tracer; Ab: antibody; P_{EX}: excitation polarizer; P_{EMA},P_{EMB}: emission polarizers perpendicularly arranged one to each other for measuring I_V and I_H, respectively.

The SF principle has been used in continuous-flow methodologies such as flow-injection analysis (FIA), known as the SF-FIA mode [11]. This involves halting the flow at the detector and measuring the slope of the linear portion of the recording reflecting the evolution of the reaction during the stoppage. However, this mode differs from the normal SF mixing technique in that sample and reagent are not mixed at a high pressure, the former does not allow fast reactions to be monitored and real-time analysis is more difficult to accomplish because of the continuous nature of the flow.

As an example of the kinetic results obtained using SF-FPIA, figure **2** shows the dynamic curves obtained for 11-nor-Δ^8-tetrahydrocannabinol-9-carboxylic acid (11-nor-Δ^8-THC-COOH) [8], which is frequently used as analogue of the major metabolite of THC, 11-nor-Δ^9-THC-COOH. These curves were obtained placing the analyte and the tracer in one drive syringe and the antibody in the other. Curves A and B were obtained simultaneously by measuring the temporal variation of the fluorescence intensity (I_F) with the emission polarizer parallel and perpendicular, respectively, to the excitation polarizer, which allows I_V and I_H values to be simultaneously monitored; curve C represent the ratio of curves A to B, and curve D the variation of P with time. As can be seen, I_V increases as the tracer-antibody complex is formed as the detector receives more radiation from the bound tracer than from the free tracer. However, I_H decreases as the polarized fluorescence emitted by the complex is filtered to a much greater extent than the unpolarized fluorescence emitted by the free tracer. Because P depends on the concentration of bound and free tracer through the I_V/I_H ratio, this ratio can also be used to determine the extent of the tracer-antibody binding reaction. The initial rate of this reaction was proportional to the slopes of curves C and D, which decreased as the analyte concentration increased.

A general feature of the SF technique is that equilibrium is reached faster than in the batch technique because of the thorough mixing of the two streams from the syringes in the observation cell. Thus, as shown in figure **2**, equilibrium is reached in about 40 s and the initial rate is measured within only 5-10 seconds.

INSTRUMENTATION

An SLM-Aminco (Urbana IL) 8000C or 8100 photon-counting spectrofluorimeter with two emission channels symmetrically arranged on both sides of the sample compartment has been used to develop several SF-FPIA methods [6-10]. The instrument is fitted with a xenon arc source and three polarizers (Glan-Thompson calcite prism type), one in the excitation light-path and the others two in

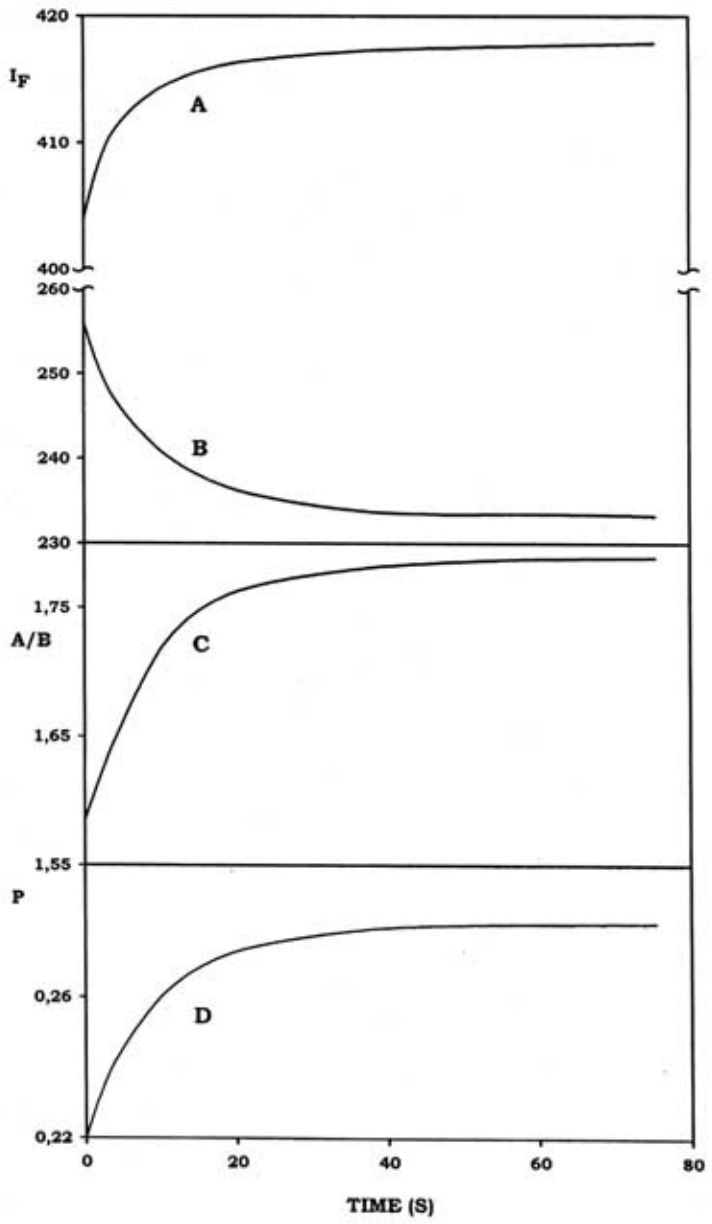

Fig. (2). Kinetic curves obtained for the cannabinoid metabolite (11-nor-Δ^8-THC-COOH) by simultaneously measuring the temporal variation of I_V (A), I_H (B), the ratio of curves A and B (C) and the degree of polarization. [analyte] = 10 ng/ml; 30-fold-diluted tracer and 15-fold-diluted antibody were used (From Ref. 8).

the corresponding emission channels of the instrument, which are placed perpendicular to each other (figure **1**). The response of both detectors is virtually the same if the emission monochromator is replaced with a filter to select the emission wavelength for each emission path of the instrument. This arrangement allows simultaneous measurements of the variation of I_V and I_H with time to be obtained, which are processed by the computer to construct the polarization-time kinetic curve.

The instrument was also furnished with an SLM-Aminco Milliflow stopped-flow module which was fitted with a 0.2-cm path-length observation cell and controlled by the associated electronics, the computer and a pneumatic syringe-drive system. The dead time of the mixing module was 2.5 miliseconds. In each run, 0.04 ml of each solution was mixed at a flow rate of 20 ml/s in the mixing chamber. The solutions in the module were kept at a constant temperature by circulating water from a thermostated tank. One of the 2 ml drive syringes of the SF module was loaded with a solution containing the analyte and the tracer and the other with the antibody solution (both were appropriately diluted with buffer). After mixing and stopping the flow,

Table 1. Analytical Performance of SF-FPIA Methods

Analyte	Sample matrix	Linear range (ng/ml)	LOD in the sample (ng/ml)	LOD using FPIA (ng/ml)
Nordiazepam	Urine	3.7-100	2.5	40
Imipramine	Serum	10-150	7.5	20
Morphine	Urine	10-300	6	25
d,l-Amphetamine	Urine	20-300	7	90
Benzoylecgonine	Urine	15-300	5	30
11-nor-Δ^8-THC-COOH	Urine	10-400	3	10
Atrazine	White wine	20-3000	6	10
2,4-D	White wine	10-1000	4	100

measurements were made as a function of time and the data processed by a computer.

FEATURES OF THE SF-FPIA METHODS

Table **1** shows some analytical features of SF-FPIA methods described for the determination of several therapeutic [6] and abused [7,8] drugs and pesticides [9,10]. All the dynamic ranges of the calibration graph and detection limits (LODs) [12] were obtained in the presence of the corresponding sample matrix. This table shows also the LODs reported by using conventional FPIA [13-19], which are higher than those afforded by SF-FPIA. The greatest difference was obtained for 2,4-D, reaching an LOD 25-times lower than that obtained by conventional FPIA.

An outstanding feature of SF-FPIA is its capability for avoiding or minimizing the background signal and the potential interferences from the sample matrix. Thus, at very low analyte concentrations, discrimination of the analytical signal from the background signal obtained in conventional FPIA is difficult, because both are static signals. However, the dynamic measurement obtained at the beginning of the reaction between the tracer and the antibody in SF-FPIA is less markedly dependent on the background signal, which does not change with time. The kinetic curves obtained for the cannabis system [8] in the absence and presence of urine matrix are shown in figure **3**. As can be seen, the initial rate is the same in both cases, but the value of *P* near equilibrium is markedly affected by the urine matrix. The same effect was found in the determination of atrazine [9] and 2,4-D [10] in white wine. Atrazine was also determined in red wine but the matrix of this sample caused a significant effect on the initial rate of the 2,4-D system, which precluded the direct determination of this analyte in red wine. However, while the conventional FPIA method for 2,4-D determination [19] was only applied to the analysis of tap water samples (a relatively simple matrix), the SF-FPIA method has shown its utility for the direct analysis of samples which have a more complex matrix such as white wine and grape juice.

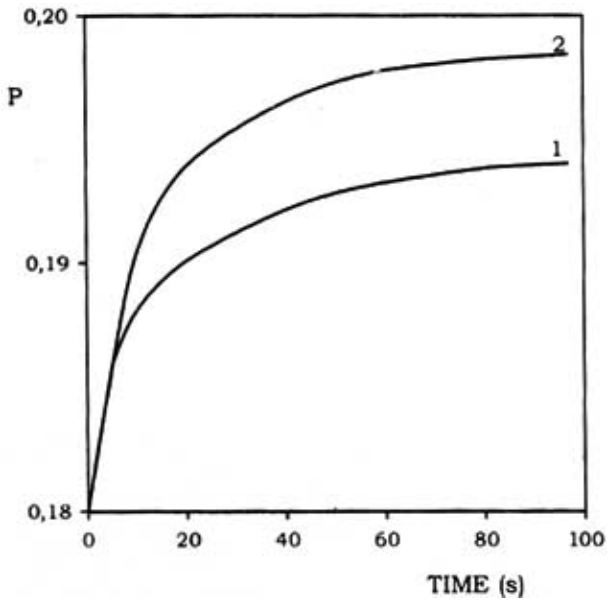

Fig. (3). Variation of the degree of polarization with time for 200 ng/ml 11-nor-Δ^8-THC-COOH in the absence (1) and presence (2) of urine matrix (10 µl) (30-fold-diluted tracer and 15-fold-diluted antibody) (From Ref. 8).

Although the use of the initial rate as the analytical parameter can improve the selectivity of FPIA, it is clear that, in addition to the type of immunoassay chosen, the selectivity afforded in an immunoassay also depends on the hapten derivative used to obtain the immunogen, which must imitate the target molecule. A general limitation of the immunoassay methods reported for atrazine is the relatively high cross-reactivity (CR) of other 1,3,5-triazine derivatives [20]. The polyclonal antibodies used in the SF-FPIA method for atrazine showed a relatively low affinity for simazine and terbutylazine but very high for terbutryn, prometryn and atraton. These results are logical taking into account that the immunogen used to obtain the antibodies contained the isopropyl and thio substituents. The CR was very high for prometryn, which contains two isopropyl substituents. Although monoclonal antibodies allow the selectivity of the atrazine determination to be improved, relatively high CR values have been obtained with other triazines [21]. Thus, a general trend in the determination of triazine residues by immunoassay [22] is to obtain antibodies with high levels of CR so that broad screening can be performed.

Table 2. Precision of SF-FPIA and Conventional FPIA

Analyte	Conc, µg/L	Method	%RSD	%RSD
			Within-assay (n=20)	Between-assay (n=8)
Amphetamine	50	SF-FPIA	3.0	2.6
	100	SF-FPIA	2.1	3.1
	300	FPIA	5.9	7.4
	600	FPIA	4.4	6.3
Benzoylecgonine	50	SF-FPIA	3.6	3.4
	100	SF-FPIA	1.9	2.4
	300	FPIA	3.0	5.5
	600	FPIA	2.2	3.2
11-nor-Δ^8-THC-COOH	20	SF-FPIA	1.4	1.8
	100	SF-FPIA	1.2	1.5
	35	FPIA	2.7	4.5
	120	FPIA	2.2	3.6

With regard to precision, as known, this analytical property was initially relatively poor in kinetic methods when the reaction rate had to be manually extracted from the recorder tracing, which decreased the precision of the results. However, this problem was overcome with the inception of electronic devices and computer that permit the automated acquisition of data as the process develops. Thus, the within-day and between-day precision values (%RSD) obtained for therapeutic [6] and abused [7,8] drugs by using SF-FPIA were generally lower than those obtained by conventional FPIA [13,14]. Table **2** shows the precision data reported for three abused drugs [8]. As can be seen, the concentration of the analytes in the kinetic method was lower than in the conventional method.

Another feature of SF-FPIA methods is their rapidity, as they do not require any incubation step before analysis. The measurement step takes only few seconds as it is carried out at the beginning of the reaction, which allows a high throughput to be obtained. Theoretically, hundreds of samples could be analysed during 1 hour. However, the practical throughput is about 60 samples/h, including the time taken to perform three replicate analyses and changeover in the system. These results show the usefulness of SF-FPIA to routine analysis.

All the reported methods were applied to the direct analysis of different samples. Table 3 summarizes the mean recoveries obtained for these analyses [6-10]. The results obtained from the analysis of serum and urine samples for the determination of therapeutic and abused drugs by the SF-FPIA methodology [6-8] were compared with those by conventional FPIA. Table **4** lists the slopes and intercepts of the linear relation between data, which were obtained by least-squares regression. The slopes were close to unity and the regression coefficients suggest good linearity, so each set of results was consistent with one another.

CONCLUSIONS

The results obtained by SF-FPIA [6-10] prove this is a useful approach for routine screening as analytical data are obtained within few seconds by measuring the variation of

Table 3. Mean Recoveries Obtained by Using SF-FPIA

Analyte	Sample	Mean recovery (%)	Reference
Nordiazepam	Urine	100.4	6
Imipramine	Serum	98.7	6
Morphine	Urine	98.5	7
d,l-Amphetamine	Urine	99.7	8
Benzoylecgonine	Urine	100.0	8
11-nor-Δ^8-THC-COOH	Urine	100.4	8
Atrazine	White wine	86.7	9
	Red wine	85.0	9
	Orange juice	92.7	9
	Tea	98.0	9
2,4-D	River water	105.0	10
	Grape juice	97.0	10
	White wine	107.7	10

Table 4. Representative Least-Squares Statistics for Comparison of the Results Provided by SF-FPIA (y) and Conventional FPIA (x)

Analyte	n	Slope[a]	Intercept[a]	r
Nordiazepam	18	0.998 (0.02)	1.9×10^{-3} (1.2×10^{-3})	0.999
Imipramine	18	0.966 (0.081)	8.8×10^{-3} (2.5×10^{-3})	0.998
Morphine	28	0.997 (0.021)	0.06 (0.25)	0.997
d,l-Amphetamine	32	0.997 (0.015)	0.054 (0.01)	0.991
Benzoylecgonine	32	0.996 (0.022)	0.062 (0.26)	0.995
11-nor-Δ^8-THC-COOH	35	1.007 (0.023)	0.949 (1.79)	0.998

[a]Mean (SD)

polarized fluorescence with time during development of immunochemical reactions. Since measurements are made before the equilibrium is reached, SF-FPIA is faster than conventional FPIA. Also, the dynamic character of analytical parameter used, which is measured at the beginning of the tracer-antibody reaction, minimizes potential interferences from the sample matrix and allows LODs obtained in FPIA to be improved. Although only the determination step of the analytical process is automated in SF-FPIA, a high sample throughput can be obtained and low sample and reagent volumes are required.

The main limitation of SF-FPIA could be found in the instrumentation required to obtain kinetic measurements, which is also related to the polarizer system necessary for measuring the variation of *P* with time. However, its use can be justified taking into account the advantages above described, although the application of SF technique to other homogeneous immunoassays based on fluorescence inhibition [23,24] and sensitized luminescence [25] requires a simpler instrumentation.

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