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Advances in drug analysis by kinetic methods[1]

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Abstract

Automatic kinetic methods are powerful tools for drug analysis as they use modern instrumentation and computers, which are essential for shortening analysis times and enhancing the quality of routine analyses. This paper reviews novel kinetic approaches to the determination of various types of drugs in bio(pharmaceutical) materials including the following: (a) a combination of the stopped-flow (SF) technique and fluorescence polarization immunoassay for the determination of drugs of abuse and opiates in urine; (b) the joint use of the SF technique and micelle-stabilized room-temperature phosphorimetry for the determination of anti-inflammatory drugs such as naproxen; (c) the use of sensitized luminescence by energy transfer processes for the kinetic determination of antibiotics such as tetracycline using the SF technique; and (d) the use of the continous addition of reagent technique for determining hallucinogenic alkaloids and phenothiazines by peroxyoxalate chemiluminescence reactions. Other advances in drug analysis by kinetic methods involving kinetometric approaches (e.g. Kalman filtering) and the use of micellar catalysis are also discussed.

Keywords: Drug analysis; Kinetic methods; Stopped-flow technique; Continuous addition of reagent technique; Kinetometric approaches; Micellar catalysis

1. Introduction

The determination of drugs and their metabolites is becoming increasingly important in the pharmaceutical and biomedical fields. Successful analyses demand sensitivity at the parts-per-billion level or lower, high selectivity and rapidity from the methods used to implement them. They require not only quality analytical results but also strict quality assurance in both procedures and data. Modern approaches to biopharmaceutical analysis rely mainly on chromatographic and immunochemical methods, which, however, are often time consuming. Immediate availability of a result is mandatory in many cases. In this respect, the worth of one analysis is inversely proportional to the time it takes:

$$\text{value} = \frac{\text{analysis}}{\text{time}}$$

Laboratory automation through modern instrumental analysis and computers and low-cost mi-

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croprocessors is essential for shortening analysis times in order to achieve high-quality routine analyses. Kinetic automatic methods are good choices for drug analyses as they permit the sensitive, selective determination of many drugs within a few seconds with no sample pretreatment in many cases. Moreover, the instrumentation required is generally very simple.

In earlier papers, we considered the role of automated kinetic-based determinations in the routine analysis of various sustances including some drugs, mainly by use of a modular stopped-flow (SF) system and the continuous addition of reagent (CAR) technique [1], and the role of the SF technique in pharmaceutical analysis [2]. Since then, new developments have arisen that enhance the kinetic determination of drugs. The most salient features of such recent developments are discussed here. Although flow injection analysis is considered to rely on kinetic principles, it is not dealt with in this paper since its instrumental and operational modes differ from those used in kinetic methods.

2. Basis of the methods

The principles and applications of the kinetic methods have been reviewed in a paper [3] and books [4,5]. Essentially, kinetic methods rely on measurements of concentration changes (detected via signal changes) in a reactant (which may be the analyte itself) with time after the sample and reagents have been mixed.

The sample and reagent can be mixed manually or automatically. Only slow enough reactions tolerate manual mixing and, even so, they are better handled automatically, not only to obtain more rapid and reproducible results, but also to increase the reaction rate in some cases. Kinetic automatic techniques are generally based on open systems, among the most popular of which are SF and CAR systems. These techniques are mandatory for fast reactions but can also be extended to slow reactions. In both cases, the kinetic curve (variation of the analytical signal with time) can be recorded immediately. The slope of the straight initial portion of the kinetic curve gives the reaction rate, which is proportional to the analyte concentration (initial-rate method). The fixed-time method, also frequently used to derive such a concentration, involves measuring the signal (the value of which depends on the analyte concentration) at a pre-set time.

These determinations can be implemented by using combinations of the SF technique with (a) fluorescence polarization immunoassay (FPIA), (b) micelle-stabilized room-temperature phosphorimetry (MSRTP) and (c) a sensitized energy-transfer fluorescence reaction, among others; all are novel strategies which have so far yielded excellent results in drug analysis. On the other hand, the CAR technique has been extended to chemiluminescence (CL) reactions as CAR chemiluminescence spectrometry (CARCL), a new approach which has proved outstanding for the analysis of drugs and other substances of analytical interest.

The use of kinetic methods in micellar media is another recent approach to kinetic-based determinations that has proved useful in drug analysis. Micelles increase the reaction rate (through micellar catalysis) and may additionally increase the sensitivity and selectivity for the analyte. On the other hand, new kinetic chemometric (kinetometric) approaches such as the Kalman filter have been developed and applied to the simultaneous determination of various compounds of pharmaceutical interest.

Recent advances in analytical techniques have facilitated measurements of very low concentrations of drugs in plasma and serum (the most frequently sampled body fluid) because drug concentrations are usually highly correlated with therapeutic effects. Urine analyses for drugs are frequently used in connection with urinary excretion and bioavailability studies. The following sections describe the most salient results obtained with the above-mentioned kinetic approaches to drug monitoring in both biological fluids and pharmaceutical preparations. The techniques used are also especially suitable for pharmacokinetic studies.

3. Drug determinations by the stopped-flow technique

The SF mode is one of the most frequently used open flow systems, particularly in kinetic and mechanistic studies of fast reactions and in kinetic multi-component determinations. Only recently (10 years ago) was the SF technique extended to routine analyses [6]. The SF technique used two propelling 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 in a mixing chamber, after which the flow is suddenly stopped by using a third, stopping syringe. Once the flow has been stopped, the analytical signal is monitored by means of a suitable detection system capable of measuring a given property of the mixed solution (e.g. absorbance or luminescence intensity). A microcomputer is used to acquire and process data. Commercially available SF equipment is very expensive. In order to facilitate routine application of this technique, most of the methods described below use a SF mixing module designed several years ago [7] which is accommodated in the detector. The mixing chamber itself acts as the observation and flow cell, and is located inside the cuvette holder of the spectrophotometer or spectrofluorimeter (the dead-time of this module is 20 ms). The kinetic curve is displayed on the microcomputer screen and the data are processed by appropriate software.

3.1. Use of conventional photometric and luminescence procedures

The use of a straightforward modular SF system for determining a number of drugs [2] was followed by new contributions. Thus, local anaesthetics such as procaine and benzocaine were determined individually and simultaneously by the rate of appearance of their condensation product with 4-dimethylaminocinnamaldehyde, which was monitored spectrophotometrically. The reaction is very fast (it takes only ca. 15 s for completion) and requires the use of the SF technique. The kinetic method used for the resolution of the mixture of both compounds allows their determination at the μg ml^{-1} level in mass ratios between 15:1 and 1:7 [8]. Benzodiazepine drugs such as bromazepam were also determined in plasma using an indirect kinetic catalytic method involving the highly selective and sensitive photometric SF measurement of bromazepam [9]. Dicoumarol, an active anticoagulant, was also determined at levels between 3 and 20 μg/ml^{-1} by SF–photometry, free of interfering substances (bromadiolone, diphacinone and warfarin) [10].

The special way in which sample and reagent are mixed in the SF technique allows the kinetic curve to be obtained simply by mixing the drug (imipramine) with its solvent [11]. Fig. 1 shows the kinetic fluorimetric profiles obtained after mixing (and stopping the flow) at various concentrations of imipramine. The phenomenon was not observed when both syringes were filled with the same solution of imipramine. Other fluorescent substances such as quinine and absorbers such as potassium permanganate and Methylene Blue gave similar results. Although no reference to this phenomenon had previously been reported, the change in the variation of the signal with time can be attributed to the rate of hydratation or solvation until a new solute–solvent equilibrium is reached. These association–dissociation processes

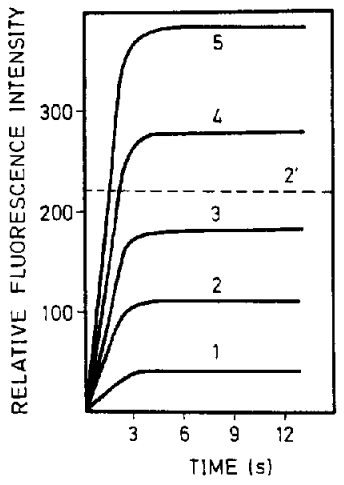


Fig. 1. Kinetic curves obtained at various concentrations of imipramine: (1) 0.10; (2) 0.30; (3) 0.50; (4) 0.75; (5) 1.00 μg ml^{-1}. (2') signal obtained with both syringes loaded with 0.30 μg ml^{-1} imipramine. λ(ex) = 290 nm, λ(em) = 400 nm. (Reproduced from [11], with permission of Elsivier Science.)

are very fast (3–5 s) and probably can only be detected when signals are acquired immediately after mixing (i.e. with the SF technique). The proposed approach was satisfactorily applied to the determination of imipramine in serum, with a very high sampling rate (measurements were performed within 5 s after mixing).

Using a different approach, mixtures of imipramine and desipramine were resolved in a single run by use of a SF–photometric method [12]. Carbamazepine, another therapeutic drug structurally similar to imipramine, was determined in serum samples by a kinetic fluorimetric–SF method based on the fluorescent reaction of carbamazepine with cerium(IV) in an acidic medium. The detection limit was 0.01 $\mu g\ ml^{-1}$ [13].

Promethazine, one of phenothiazine derivatives most frequently used as an antihistaminic, was determined in pharmaceutical preparations by monitoring its rate of oxidation to the fluorescent sulphoxide. The most salient feature of this method is that no oxidant need be added to the reaction medium. Thus, both syringes were loaded with the same drug solution in order to avoid sample dilution. Thus, again, the special features of the SF mixing technique (rapid, thorough mixing and pressure-promoted collisions between promethazine and dissolved oxygen molecules) allowed the oxidation to be rapidly completed [14].

Some antibiotics have also recently been determined by SF methodology. The method for ampicillin in serum excels its equilibrium counterpart, particularly as regards analysis time. The well known fluorescence methods involving hydrolysis (to open the β-lactam ring) and the reaction of mercury(II) chloride with its mercapto group, which entails waiting for about 30 min before measurements can be made, are dramatically expedited by the addition of hydrogen peroxide and the features of the SF technique, avoiding the prior hydrolysis step [15]. Other advantageous methods for aminoglycoside antibiotics such as amikacin [16] and tobramycin [17] in pharmaceutical preparations without sample pretreatment have also been reported. Bromhexine, a mucolytic drug widely used in conjunction with antibiotics such as penicillins, was determined in only 0.5–1 s by using SF–photometry and the initial-rate method. Cephalosporins and penicillins in a 100-fold excess had no effect on the analyte determination [18]. Also, clavulanic acid, which increases the reactivity of some of the above-mentioned β-lactam antibiotics, was determined in a few seconds by reaction with imidazole using an SF–photometric method that was applied to pharmaceutical and serum samples with no sample pretreatment, with good results [19]. Because clavulanic acid is usually included in formulations containing amoxycillin, where the antibacterial activity of the latter is increased by the acid, the determination of both drugs in mixtures is of great interest. To this end, an SF–fluorimetric method based on the different rate of formation of both fluorescent derivatives in the presence of cerium(IV) was developed. The method was applied to the simultaneous kinetic determination of both drugs in pharmaceutical formulations with an RSD of less 3% [20]. The individual and joint SF–spectrofluorimetric determination of neomycin and tyrothricin in pharmaceutical preparations was also accomplished with satisfactory results [21].

Ephedrine and phenylephrine, two sympathomimetic agents, exhibit a disparate kinetic behaviour when the corresponding aldehydes formed by oxidation of these compounds react with 2-methylbenzothiazoline-2-one hydrazone (MBTH), a reagent previously used for equilibrium measurement of these drugs. An SF method avoids the extraction step in the determination of ephedrine and testifies to the effectiveness of kinetic methodology in conjunction with the SF mixing technique to shorten the time required for measurements. Both compounds can be determined individually and jointly with good results (the mixture cannot be resolved by equilibrium methodology) [22].

The joint use of a diode-array detector and the SF mixing technique allows the simultaneous kinetic resolution of mixtures by fast reactions, whatever the relative values of the rate constants involved. Such is the case with methotrimeprazine and thioridazine, two phenothiazines commonly used as tranquilizers, which were determined individually and in mixtures. The ensuing methods

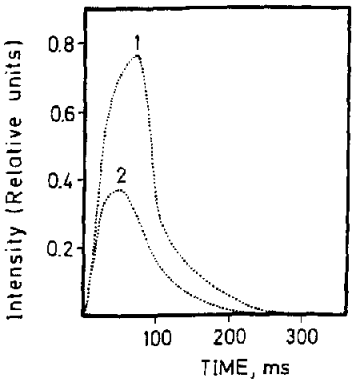


Fig. 2. Effect of penicillin G on the CL luminol–iodine system, (1) in the absence and (2) in the presence of the antibiotic. [Penicillin G] = 2×10^{-5} M. (Reproduced from [25], with permission of Elsevier Science.)

are based on measurements of the rate of oxidation of each compound by iron(III) to give the corresponding phenothiazyl free radical, which absorbs maximally at two different wavelengths (564 nm for methotrimeprazine and 634 nm for thioridazine). The reaction rate of each analyte was measured simultaneously within about 5 s and the proportional equation method was applied to initial-rate and signal amplitude measurements [23].

A combination of the SF technique and chemiluminescence (CL) reactions was recently used to obtain the whole CL profiles [24] (which is impossible with continuous and flow-injection systems owing to the rapidity of the CL signal), for which CL formation and decay rates can be related to the analyte concentration with a higher precision and selectivity relative to the peak light intensity or the area under the light emission–time curve. New approaches have been developed by use of SF–CL spectrometry (SF–CLS). One is the determination of penicillins by their inhibitory effect on the luminol–iodine system [25]. The CL profile of the system in the presence and absence of penicillin G is shown in Fig. 2. The ensuing method allows the determination of penicillins over the range 1×10^{-6}–1.2×10^{-4} M (RSD $\approx$ 2%) at a sampling frequency of 120 h^{-1}; it is precise and rapid, uses little sample, is amenable to automation and compares favourably with its batch counterpart, which involves a CL titration assay. The method allows the determination of penicillins in pharmaceutical samples with no interference from excipients commonly accompanying penicillins. Another interesting application of SF–CLS is in the determination of periodate and tartaric acid, and of the latter in pharmaceutical preparations [26]; the dynamic range for tartaric acid is 2×10^{-6}–5×10^{-5} M and the method compares favourably with existing alternatives in terms of sensitivity and sampling frequency.

3.2. Novel luminescence approaches

The experience gathered in using the above-described methodology has prompted the development of new stopped-flow luminescence approaches that are briefly described below.

3.2.1. SF–fluorescence polarization immunoassay

Fluorescence polarization immunoassay (FPIA) is a homogeneous immunoassay based on the difference in molecular volume of a small fluorescent-labelled antigen or hapten when free and when bound to a large antibody. The competitive reactions between the antigen (drug-analyte, Ag) antigen labelled fluorescein (tracer, Ag*) and the antibody (Ab) can be formulated as follows:

$$\begin{matrix} Ag & & Ag\text{–}Ab \\ & + Ab \rightarrow & \\ Ag^* & & Ag^*\text{–}Ab \end{matrix}$$

The tracer, which competes with the analyte for the antibody, will only exhibit polarized fluorescence when bound to the antibody owing to its higher molecular volume, which is inversely proportional to the analyte concentration. The polarized fluorescence is usually measured at equilibrium, even though it can also be measured during the reaction by monitoring the tracer–antibody (Ag*–Ab) system. One possible reason for the lack of FPIA kinetic methods is that these complex reactions are so fast that kinetic measurements require the SF mixing technique. Furthermore, this technique allows methods to be readily automated since reactant manipulation is minimal and measurements are performed shortly after mixing.

Fluorescence polarization is usually expressed as the degree of polarization, $P = (A/B - 1)/(A/B + 1)$, where A and B are the fluorescence intensities measured with the emission polarizer parallel and perpendicular, respectively, to the excitation polarizer.

The only requirement for application of kinetic methodology in FPIA is the availability of instrumentation that permits simultaneous measurements of the variation of A and B with time, to be processed by a computer in order to construct a polarization–time kinetic curve. A T-spectrofluorimeter with two emission channels symmetrically arranged on both sides of the sample compartment and the emission polarizers perpendicular to each other is suitable for this purpose. Measuring the variation of P with time (dP/dt) allows one to obtain the reaction rate of the tracer–antibody complex. An SLM 8000 C photon-counting spectrofluorimeter (Urbana, IL, USA) furnished with an SLM-Aminco Milliflow stopped-flow module fitted with a 0.2 cm pathlength observation cell was used to develop several SF–FPIA methods for drug analysis; the dead time of the mixing module was 2.5 ms. One of the 2 ml drive syringes of the SF module was loaded with the drug solution and the labelled antigen and the other syringe with the antibody (both were appropriately diluted with buffer). After mixing and stopping the flow, measurements were made as a function of time and the data were processed by a computer.

The new methodology was applied to the direct determination of drugs of abuse (amphetamine, cocaine and cannabinoid metabolites) in urine [27]. Fig. 3 shows the kinetic curves obtained for the cannabinoid metabolite 11-nor-Δ^8-tetrahydrocannabinol-9-carboxylic acid (11-nor-Δ^8-THC-COOH) by simultaneously measuring the temporal variation of the fluorescence intensity (IF) with the emission polarizer parallel (A) and perpendicular (B) to the excitation polarizer; curve C in Fig. 3 represents the ratio of curves A to B, and curve D the variation of the degree of polarization with time. Because P depends on the concentrations of bound and free tracer through the A/B ratio, the ratio can also be used to determine the extent of the antibody–tracer binding reaction. Thus, the initial rate of this reaction ($V_{A/B}$ or V_P) is proportional to the slopes of curves C and D, which decreases as the analyte concentration increases. As shown in Fig. 3, equilibrium is reached within 40 s and the initial rate can be measured within only 5–10 s.

SF–FPIA methodology enhances the determination capabilities of conventional FPIA for drugs of abuse, (a) by lowering the limits of determination and detection (Table 1) and raising the within- and between-array precision; and (b) by reducing the dependence on the background signal, which does not change with time owing to the dynamic character of the kinetic parameter (see Fig. 4). The initial rate measured from the slope of the P vs. time plot for 200 $\mu g\ l^{-1}$ of 11-nor-Δ^8-THC-COOH is the same in the absence (curve 1) and presence (curve 2) of the urine matrix (10 μl).

Similar results were obtained in the SF–FPIA determination of opiates (e.g. morphine) in urine. The detection limit was lower and measurements were independent of the background signal for the

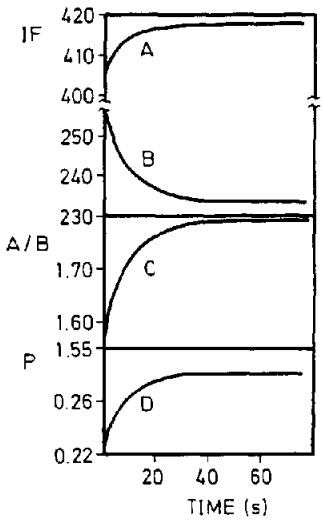


Fig. 3. Typical kinetic curves obtained for the cannabinoid metabolite by simultaneously measuring the temporal variation of the fluorescence intensity (IF) with the emission polarizer parallel (A) and normal (B) to the excitation polarizer. C is the ratio of curves A to B and D the variation of the degree of polarization with time. [11-Nor-Δ^8-THC-COOH] = 10 $\mu g\ l^{-1}$; 30-fold-diluted tracer and 15-fold-diluted antibody were used. (Reproduced from [27], with permission of the American Clinical Association.)

Table 1
Comparison of determinations of drugs of abuse ($\mu g\ l^{-1}$) by SF–FPIA and conventional FPIA (reproduced from [27], with permission of the American Clinical Association)

Analyte	Dynamic range		Detection limit	
	SF–FPIA	FPIA	SF–FPIA	FPIA
D,L-Amphetamine	20–300	150–3000	7	90
Benzoylecgonine	15–300	300–5000	5	30
11-Nor-Δ^8-THC-COOH	10–400	25–150	3	10

sample matrix, in contrast with the conventional FPIA method [28]. Therapeutic drugs (benzodiazepines and tricyclic antidepressants) in biological fluids were also assayed [29] in order to assess the advantages of this novel approach over conventional equilibrium methods; the former were found to provide better detection limits and precision than conventional FPIA.

Kinetic methodology possesses other major advantages, namely: (a) determinations are direct and rapid (the time required to obtain analytical data is only about 5–10 s), so sampling frequencies are usually high (typically above 60 h^{-1}) and long incubation periods (60 min) are avoided; and (b) the reactant manipulation is considerably reduced. All these features make automated SF–FPIA methods especially suitable for routine analyses.

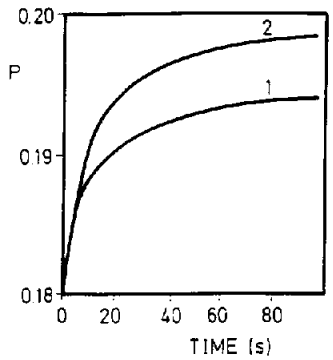


Fig. 4. Variation of the degree of polarization with time for 200 $\mu g\ l^{-1}$ 11-Nor-Δ^8-THC-COOH in (1) the absence and (2) the presence of urine matrix (10 μl) (30-fold-diluted tracer and 15-fold-diluted antibody). (Reproduced from [27], with permission of the American Clinical Association.)

3.2.2. SF–T-format spectrofluorimeter for simultaneous kinetic analyses

The above described T-format spectrofluorimeter furnished with an SF module was used for the direct resolution of a mixture of two drugs, neomycin and promethazine, an antimicrobial and an antihistaminic, respectively, which are found together in some pharmaceutical preparations [30]. The T-format configuration, which involves two emission paths symmetrically arranged on both sides of the sample compartment, allows one to monitor the development of the reaction of neomycine with *o*-phthalaldehyde in the presence of *N*-acetylcysteine and the oxidation of promethazine by dissolved oxygen without the need for an oxidant such as hydrogen peroxide since both reaction products have similar excitation wavelengths but different emission wavelengths. The absence of overlap in the emission maxima allows one to obtain kinetic data from each individual system, even if the two systems possess similar rate constants and their reactions are fast. This is the first reported application of kinetic methodology based on reaction-rate measurements to a simultaneous fluorimetric determination using a dual-channel instrument.

The kinetic curves simultaneously obtained for promethazine ($\lambda_{am} = 370$ nm) and neomycin ($\lambda_{em} = 470$ nm) exhibit a disparate kinetic behaviour: whereas neomycin reaches equilibrium within only 3–4 s, promethazine takes about 40 s. However, the high initial rates of both systems justify the use of the SF mixing technique. The ensuing method allows the resolution of mixtures containing neomycin and promethazine in ratios between 8:1 and 1:9 with good selectivity and acceptable sensitivity (at the $\mu g\ ml^{-1}$ level). It

was validated in the simultaneous determination of both drugs in a pharmaceutical sample also containing hydrocortisone acetate and retinol.

3.2.3. SF–energy-transfer sensitized luminescence

Kinetic methodology has also been applied to complex-formation reactions involving energy-transfer processes. One of the better known of such processes is the intramolecular energy transfer from tetracycline to europium ion. Tetracycline excited at 392 nm undergoes inter-system crossing to its triplet state and the associated energy is transferred to the 4f level of europium ion, which gives a characteristic line-type emission at 612 nm. Analytical methods for the determination of the antibiotic with a very low detection limit (10 ng ml^{-1}) were proposed that were very recently improved by adding Triton X-100 to the aqueous solution (the micelles cancel the quenching of coordinated water molecules and protect the Eu(III)–tetracycline chelate against non-radiant processes).

The original method for the determination of tetracycline in the presence of a surfactant was improved (a) by adding thenoyltrifluoroacetone (TTA), which exerts a synergistic effect (it removes water molecules from the coordination sphere of the lanthanide ion) and (b) by using the SF mixing technique to monitor the formation rate of the Eu(III)–tetracycline–TTA ternary complex in the micellar medium [31].

The joint use of Triton X-100 and TTA as the second ligand is neccessary to ensure high sensitivity (see Fig. 5). The kinetic curves were obtained by placing Eu(III) and TTA in one of the syringes and tetracycline plus Triton X-100 in the other. As can be seen, the rate in the present of TTA and surfactant (curve 3) was ca. ten times higher than that obtained in the absence of TTA (curve 2). The slope of curve 3 was linearly proportional to the concentration of tetracycline over the range 20–1000 ng ml^{-1}.

The SF technique is necessary to obtain the initial-rate measurements since the formation of the ternary complex is very fast. In addition, it offers some advantages such as reduced reactant volumes and manipulation and ease of automation, which avoids the need for blank background

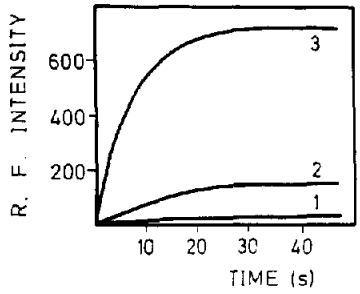


Fig. 5. Kinetic curves obtained for: (1) Eu(III) + tetracycline, (2) Eu(III) + tetracycline + 1% Triton X-100, (3) Eu(III) + tetracycline + TTA + 0.01% Triton X-100. [Tetracycline] = 1 μg ml^{-1}. [Eu(III)] = 6.6×10^{-4} M (curve 1), 5×10^{-5} M (curve 2) and 2×10^{-4} M (curve 3). [TTA] = 5.4×10^{-5} M (curve 3). (Reproduced from [31], with permission of Elsevier Science.)

fluorescence corrections and allows the analytical results to be obtained promptly (within about 5 s). The ensuing method was validated in the determination of tetracycline in various spiked human sera by using 0.4 ml of untreated sample (no precipitation of serum protein was required) with a mean recovery of 98.8%.

The above-described method was extended to the determination of tetracycline–doxycycline mixtures by their differential kinetic behaviour. The kinetic curves show that the formation rate of the Eu(III)–doxycycline–TTA ternary complex (in the presence of Triton X-100 micelles) is lower than that for tetracycline and that no synergistic effect is present. This allows both antibiotics to be simultaneously determined using the proportional equation method with tetracycline-to-doxycycline ratios between 5:1 and 1:7 in the presence of other antibiotics such as penicillins (ampicillin, amoxycillin, doxacillin) and cephalosporins (cephadin, cephalexin, cephaloridin), which do not interfere at concentrations at least 5 times higher than those of the analytes [32]. Direct analysis (without protein precipitation) of serum samples spiked with different amounts of tetracycline and doxycycline in mixture was accomplished with excellent results (RSD < 2.5% and a mean recovery of about 97% for each analyte). Nalidixic acid and norfloxacin in serum samples were also determined by direct analysis with satisfactory results using terbium-sensitized fluorescence [33].

3.2.4. SF–micelle-stabilized room-temperature phosphorimetry (MSRTP)

Kinetic methodology is widely used in phosphorimetry for determining the lifetime of excited triplet states, yet it has never been used so far for analytical purposes by measuring the rate of formation of a phosphorescent system. This approach is especially useful when the phosphorescent signal is unstable or takes a long time to stabilize, which hinders its use in routine analyses for drugs. As the rate of formation of a phosphorescent system is usually very high, kinetic data cannot be obtained by using the batch mode but only with the SF mixing technique.

MSRTP in aqueous solutions containing a heavy atom was chosen for the purpose as it was better suited to the SF technique than the mode using a solid support (SSRTP). This latter has serious constraints that are circumvented by SF–MSRTP [34]. This advantageous combination has been demonstrated in the rapid kinetic determination of naproxen ((+)-2-(6-methoxy-2-naphthyl)propionic acid, a drug with anti-inflammatory, analgesic and antipyretic properties, in serum samples. The method involves the interaction of naproxen with thallium(I) ion in the presence of SDS and sulphite ion (to remove dissolved oxygen), which causes a sharp increase in the phosphorescence signal that reaches equilibrium in only 2 s. The slopes of the phosphorescence–time curves (proportional to the naproxen concentration) were determined within ca. 1 s and the blank signal was found to be negligible [35].

Recently, a batch non-kinetic determination method based on the previous system was used for the determination of naproxen [36]; the procedure takes ca. 30 min to remove oxygen and a further 10 min to obtain a stable signal before measurements can be made. The SF methodology avoids this delay since the pressure exerted by the instrumental system favours the interaction of oxygen molecules with sulphite ions and hence removal of the former. The results can be obtained shortly after the drug and reagent have been mixed.

4. Drug determinations by the continuous addition of reagent (CAR) technique

This technique is a fairly recently reported open system approach based on the continuous addition of a reagent solution at a constant rate to another solution containing the analyte to be determined [37]. The chief advantages of this approach are that the kinetic response curve can be readily obtained from both fast and slow reactions (for which the first-order condition is commonly used) since the reaction can be made second order [38] with very simple instrumentation. The typical instrumental set-up consists of (1) an addition unit with an autoburette, a magnetic stirrer and a thermostated vessel (the reaction cell); (2) a detector (photometric, fluorimetric or chemiluminescence); and (3) a computer unit for data acquisition and processing. The second-order kinetic profile of the signal vs. time (or volume added) response includes a straight portion the slope of which is proportional to the analyte concentration (maximum-rate method) which is equivalent to the initial-rate method used for pseudo-first-order kinetic responses in the SF technique [38].

Several drugs have been determined by using the CAR technique with photometric [39,40] and fluorimetric [41] detection. The next section discusses only the most recent methods for drug analysis using "conventional" CAR or a novel CAR approach adapted to chemiluminescence reactions.

4.1. Conventional photometric and fluorimetric procedures

Oxazepam [42] and nitrazepam [43], two well known psycotherapeutic drugs of the benzodiazepine family, were determined by continuously adding alkaline 1-naphthol to a reaction vessel containing previously hydrolysed drug solution and sodium nitrite to give the corresponding azo dyes, which were monitored by absorbance measurements at 530 and 600 nm, respectively, against time. The methods outperform their conventional equilibrium counterparts as they permit one to distinguish the intact drug from its hy-

drolytic degradation product and are suitable for the determination of oxazepam and nitrazepam in pharmaceuticals and the oxazepam in urine samples, at a sampling frequency of 60 h^{-1}.

Determinations of corticosteriods (cortisone, hydrocortisone, prednisone, prednisolone, dexamethasone and betamethasone) in biological and pharmaceutical samples remain of great interest and a large variety of methods continue to be developed, many of which are based on the Blue Tetrazolium (BT) and Porter–Silver reactions. The CAR technique was used for the determination of above-mentioned corticosteriods using both reactions with photometric detection [44]. Judging from the results shown, the technical problems associated with these reactions (e.g. the long incubation time (30–60 min) at high temperatures (50–100°C) are avoided and the CAR method is simpler, faster and more sensitive than its equilibrium counterparts; also, it lends itself readily to routine analyses for corticosteriods. The CAR–BT method compares favourably with other recent alternatives using the same chemical system (SF and FIA methods included). Its performance was evaluated in recovery experiments and the analysis of commercially available formulations.

One of the more frequently used flourimetric methods for the determination of morphine is based on the classical oxidative dimerization of this drug by alkaline potassium hexacyanoferrate(III) to form a highly fluorescent dimer (pseudomorphine). However, this method poses problems arising from the instability of the reaction products and undesirable interactions between them and excess reagent (e.g. quenching due to excess hexacyanoferrate(III) in the oxidation of morphine). These problems are also encountered in batch and even flow techniques.

The CAR technique avoids the above problems since the reagent is continuously added from the autoburette, thereby increasing the fluorescence intensity [45]. The oxidation reaction is fairly rapid but can be followed using this technique by spectrofluorimetric monitoring of the reaction rate of the pseudomorphine formed; the kinetic curve can be obtained and processed by the data acquisition system in a single run. The CAR method for the automatic determination of morphine is also very sensitive (detection limit of 2.76 ng ml^{-1}), precise (RSD $\approx$ 2%), highly selective and rapid (48 samples h^{-1}). It was applied to the determination of morphine in urine, which is of interest in order to confirm overdosing in addicts or doping in sport events; the procedure used for this purpose uses a novel sorption–desorption step to isolate morphine from whole urine with recoveries of 85–100%.

4.2. CAR–CL spectroscopy

The adaption of the CAR technique to CL reactions was recently approached [46]. CAR–CL spectroscopy (CLS) has some advantageous features, namely: (a) it facilitates thorough, reproducible mixing of sample and reagent; and (b) it allows recording of the whole CL response curve. These features are similar to those of SF–CLS described above. However, the CAR–CLS technique has one additional advantage, viz. it allows the rate of the CL reaction to be altered (decreased on account of its second-order nature) so that it can be monitored with a straightforward data-acquisition system.

Another relevant additional feature is that, because of the special nature of CAR–CLS, it can be used as an effective means for suppressing background emission in peroxyoxalate (PO)–CL reactions, thereby providing an effective, simple method for the determination of phenothiazine derivatives [47]. The experimental set-up for this

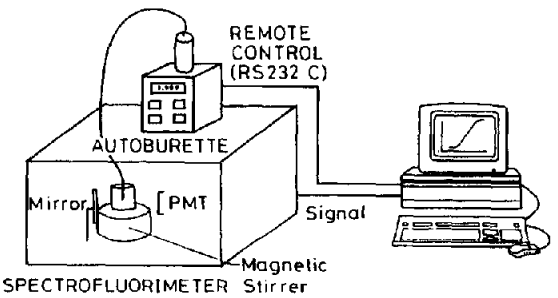


Fig. 6. Experimental set-up of the continuous addition of reagent technique for the PO–CL determination of drugs. (Reproduced from [47], with permission of the American Chemical Society.)

technique is shown schematically in Fig. 6. The nature of the background emission observed in PO–CL reactions has been thoroughly studied; the formation and decomposition of CL reaction intermediates seem to be responsible for the peroxyoxalate background CL. The bis(2,4,6-trichlorophenyl) oxalate (TCPO)–H_2O_2 system was used to develop an analytical method of this type and TCPO solution was added to the reaction vessel containing the drug to be analysed and the oxidant at a rate of 6.5 ml min^{-1} with continuous stirring. Data were acquired simultaneously at a rate of 20 ms per point (the maximum reaction rate was measured within about 1 s). These conditions favour suppression of background emission and provide a zero dead-time between mixing of TCPO and hydrogen peroxide and reaction with the fluorophore (a phenothiazine derivative), in contrast with those flow systems where TCPO and hydrogen peroxide are generally mixed first and the mixed stream is then reacted with the fluorophore. This increases the efficiency of the PO–CL reaction and avoids the typical problem of the instability of aqueous TCPO. The best results among the drugs tested were obtained for acepromazine and propiopromazine (with detection limits of 2.2 and 2.7 ng ml^{-1}, respectively), and compared favourably with those provided by existing alternatives in terms of sensitivity, precision and sampling frequency. The determination of acepromazine (of widespread use in veterinary medicine) in horse plasma was used to validate this new approach to analyse real samples.

This approach and 2.4-dinitrophenyl oxalate (DNPO) instead of TCPO was used for the determination of hallucinogenic alkaloids in the first reported application of a PO–CL reaction [48]. Fig. 7 shows the kinetic profiles obtained. DNPO gives one of the highest CL quantum yields; however, the background emission is also higher than with TCPO, which dictates the detection limit achieved. Furthermore, the CL half-life is shorter for DNPO than for TCPO owing to the strong electron-withdrawing effect of the nitrogroup relative to chlorine substituents, which is a serious constraint in static and flow determinations. All these drawbacks were overcome by using the

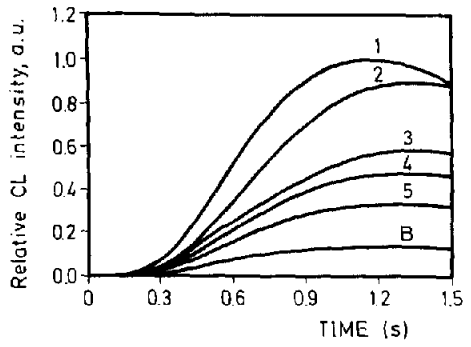


Fig. 7. Relative CL intensity vs. time profiles for different hallucinogenic alkaloids; (1) harmalol; (2) harmaline; (3) harmane; (4) harmol; (5) harmine; (B) blank. (1) and (2) 15 ng ml^{-1}; other hallucinogenic alkaloids, 1.0 μg ml^{-1}. (Reproduced from [48], with permission of Elsevier Science.)

CAR technique to implement this PO–CL reaction for the kinetic determination of six alkaloids including harmaline, which was quantified in plasma samples to validate the method. As can be seen in Table 2, the sensitivity achieved for harmaline and harmol was 2–3 orders of magnitude higher than those of previously reported methods [48].

5. Drug determinations by kinetometric approaches

Although drugs in mixtures are usually determined chromatographically, when the mixture is not very complex (two or three components), simpler, faster techniques are to be preferred. In this respect, kinetic multicomponent methods al-

Table 2
Comparison of the dynamic linear range and detection limit for β-carboline alkaloids afforded by the CAR–(PO–CL) method and other approaches (reproduced from [48] with permission of Elsevier Science)

Approach	Dynamic linear range/LOD
HPLC	ng–μg level
Adsorptive stripping voltammetry	0.2–1.6 μg ml^{-1}
Spectrofluorimetry	LOD: 16–55 ng ml^{-1}
CAR–(PO–CL) method	1–150 ng ml^{-1} LOD: 0.25–0.30 ng ml^{-1}

low the variation of the analytical signal yielded by each species to be "separated" on a different time-scale, thereby avoiding physical separation in space [4].

Current trends in kinetic chemometric approaches rely on multi-point curve-fitting methods that take full advantage of the information provided by experimental data [3] thanks to the high computational power routinely available. One of the most useful choices for accomplishing kinetic determination of drugs in mixtures is the Kalman filter, using both linear and non-linear (extended Kalman filter) modes.

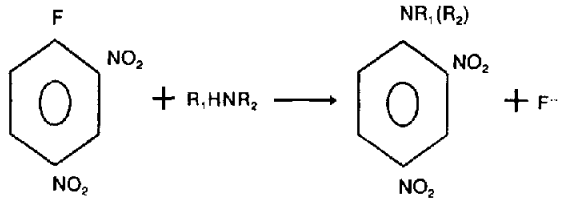
Thus, binary mixtures of cortisone and hydrocortisone were satisfactorily resolved by using the Kalman filter [49]. The reaction used was that of Blue Tetrazolium (BT); although cortisone reacts faster with BT than does hydrocortisone, the rate difference between the two drugs is small (the pseudo-first-order rate constant of cortisone to hydrocortisone is only 1.8). Therefore, the Kalman filter is especially useful for the analysis of closely related drugs exhibiting a similar kinetic behaviour. In those cases, conventional multicomponent methods (e.g. those with logarithmic extrapolation and the single point and proportional equations) fail, as demonstrated in the determination of the above-mentioned corticosteroids in mixtures, where only the Kalman filter provided good results [49]. In fact, the errors made from absorbance measurements were less than 10% in all instances and mixtures of the two corticosteroids with cortisone-to-hydrocortisone concentration ratios from 2.5:1 to 1:3.3 were successfully resolved by the kinetometric Kalman filter approach.

6. Drug determinations by micellar kinetic approaches

Micellar media have been widely used to facilitate or imporve the determination of a large number of drugs by use of various procedures such as micellar liquid chromatography [50], flow injection [51], acid–base potentiometric titration [52] and micellar electrokinetic chromatography [53].

However, micelles started to be used in kinetic methods only recently [54]. Most such methods rely on the ability of micelles to accelerate reactions. Micellar catalysis is useful not only for accelerating slow reactions (and hence adapting the time-scale of a kinetic method to the specific instrumental requirements with a view to automation), but also for enhancing analytical properties, improving selectivity, increasing drug solubility or simplifying the operational procedure.

Among the typical reactions used for the kinetic determination of drugs by micellar catalysis are those of 1-fluoro-2,4-dinitrobenzene (FDNB) with amines, phenols, thiols and hydrazines to yield dinitrophenyl derivatives and fluoride ion:



Scheme 1.

These reactions are slow and usually require heating to proceed at an acceptable rate. Also, additional steps for hydrolysis of excess FDNB and extraction of the dinitrobenzene product for photometric measurement are required. In the presence of cationic and non-ionic micelles, the reactions are considerably accelerated (by concentration of the analyte substrate on the micellar surface) and the above-mentioned problems are overcome. Cefalexine, sulphamethazole and various amino acids were thus successfully determined in the presence of cetyltrimethylammonium bromide (CTAB) by monitoring the fluoride produced using a fluoride ion-selective electrode [55]. The enhancing effect of CTAB micelles was also exploited for the kinetic potentiometric determination of acetaminophen in commercially available formulations [56]. The reaction rate of this drug with FDNB was increased 130-fold in the presence of the cationic surfactant. Moreover, the method is not subject to any interferences from excipients or from cloudy or coloured solutions.

Isoniazide was also determined by reaction with FDNB using a kinetic spectrophotometric

stopped-flow injection method [57]. The use of an automated method is advisible since the FDNB reagent is difficult to handle (it is a vesicant and hydrolyses in alkaline solutions).

7. Conclusions

Automatic kinetic methods based on open systems such as those reviewed in this paper are useful choices for drug analyses (expecially individual species or simple mixtures (2–3 components) of drugs) on account of their simplicity and rapidity and the high quality of results achieved in routine determinations. Future prospects may include (1) new kinetic automatic approaches, (b) development of new kinetometric methods using neural networks in multicomponent drugs analysis and (c) use of organized media containing micelles and aggregates of different types to facilitate and/or improve drug determination methods.

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