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Review

Recent progress of flow-through optosensing in clinical and pharmaceutical analysis

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ABSTRACT

The applications of flow-through optosensing in clinical and pharmaceutical analysis in the last decade are here reviewed. A classification of the flow methodologies employed (conventional flow injection analysis, multicommutated flow analysis and sequential injection analysis) is introduced and their principal characteristics explained. The fundamentals of flow-through optosensing together with the main detection techniques are described and, later, the main applications of these flow methods are highlighted, paying special attention to the recent incorporation of new detection techniques in optosensing and to the design of multiparameter sensors. Finally, the advantages and disadvantages of the different flow methodologies here described are critically discussed.

Contents

1. Introduction

The inception of flow analysis have proved to be an excellent tool for handling solutions and, consequently, performing methods related to wet chemical analysis. Especially prominent among flow techniques is Flow Injection Analysis (FIA), proposed in 1975 by Ruzicka and Hansen [1], which initiated a field of research that would, over more than three decades result in close to thousand of publications in the international scientific literature. In general, in this technique, the sample is introduced by using a propelling system and processed under reproducible conditions, allowing its isolation from the environment and, therefore, avoiding external contamination [2,3]. Different devices have been employed, although the six-port rotary valve has been the most widely used

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injection system. Inherent features of FIA are low consumption of solutions, good accuracy and precision of results, high sample throughput, and overall robustness.

The main aims of recent developments in flow analysis have been to progressively achieve lower reagents consumption, higher repeatability, complete automation of the analytical procedure and miniaturization. In this sense, the main flow methodologies employed in recent years, in addition to conventional FIA, have been sequential injection analysis (SIA), multicommutated flow techniques and lab-on-valve flow systems, each method presenting advantages and drawbacks when compared to each other.

SIA was introduced by Ruzicka and Marshall in 1990 as a following generation in the development of flow injection technique [4]. The main components of a SIA manifold are a multi-position selection valve and a bi-directional syringe pump, both automatically controlled by a computer and the main improvements when compared to FIA are its higher robustness and automation, together with lower sample and reagent consumption.

In addition to SIA, recent modifications in flow analysis include the concept of multicommutation, introduced by Reis et al. [5] in order to obtain complete automation, miniaturization, lower reagents consumption and higher repeatability and versatility. It consists in the employment of discrete commutation devices (solenoid valves, for example) to build up dynamic manifolds that can be easily reconfigured by software. This approach greatly increases the versatility of the flow systems since each analytical step can be independently implemented. The main differences between multicommutation and the other flow methodologies is that insertion volumes are replaced with insertion times and the possibility of establishment of multiple reaction interfaces by means of the binary sampling approach [6]. Although the multicommutation concept has been applied in the development of different flow methodologies [2], we will focus on Multicommutated Flow Injection Analysis (MCFIA), which is the only one that has been used in flow-through optosensing with pharmaceutical applications. MCFIA systems are typically constituted by a peristaltic pump and a set of three-way solenoid valves, automatically controlled by appropriate software, which can be arranged creating a flow network. Each valve can adopt two positions, “ON” and “OFF”, being the whole system assimilated to an electronic circuit with a variable number of active nodes.

Although all flow systems here described offer inherent outstanding advantages, already highlighted, they have to be complemented by selective and sensitive detection techniques, additional separation devices and clean-up steps when analyzing complex matrices or determining more than one analyte. In this sense, solid phase spectroscopy (SPS) plays an important role. In SPS, the species of interest is sorbed on an appropriate solid support and its detection is performed directly on the solid phase; this way, great selectivity and sensitivity are achieved. These inherent features make SPS an interesting option for its implementation in automatic flow systems, as it could be used for the retention of the reagents, the target analyte or even the product of the reaction. The coupling between SPS and flow systems is the so-called flow-through optosensors, a versatile tool for the analysis of one or several analytes in complex samples. The optosensors developed in the last decade with clinical and pharmaceutical applications will be here reviewed.

2. Flow methodologies classification

The basic components of any flow system include: propelling system, device/s for the introduction of solutions into the system, flow-through cell and detector. As it has been previously stated, three flow methodologies have been used in pharmaceutical flow-through optosensing and will be here described (Fig. 1).

2.1. Conventional FIA system

The basic components are a peristaltic pump to propel the sample and reagents, a series of plastic tubes to carry the solutions, injection valves to introduce constant volumes of sample and reagents in the system and the detector, being the valves usually employed the manually-controlled six-way rotary valves [7]. The main advantages of a FIA system are the rapidity, repeatability, versatility and automation.

2.2. SIA system

In order to increase the automation and versatility of the FIA systems, SIA was introduced as a following generation in the development of flow injection technique. The main components of a SIA manifold are a multi-position selection valve and a bi-directional syringe pump, both automatically controlled by a computer. The use of the multi-position valve makes it unnecessary the use of additional valves, such as in conventional FIA systems, where several valves are needed. The main advantages of the SIA are very simple manifolds, minimum solutions wasting, automation, high repeatability, possibility of using very low volumes of solutions and very robust systems.

2.3. MCFIA systems

The key element in MCFIA is the solenoid valve, usually 3-way solenoid valves. Each valve acts as an independent switch and the whole system is automatically controlled by a computer. These systems are similar to an electronic circuit, with a variable number of active nodes, which present two different positions: “ON” and “OFF”, allowing the effective control of sample and reagents dispersion and widening the scope of applications in flow analysis. Compared to conventional FIA, the insertion volume measurement is replaced with time measurement. Hence, the repeatability in multicommutation systems is associated not only with the stability of the peristaltic pump, but also with the precision in time measurement, obtaining better repeatability. The main advantages of multicommutation are miniaturization of the manifolds, reduced sample and reagents consumption, automation, high repeatability, economy and versatility.

3. Solid phase spectroscopy

The introduction of a solid support in conventional FIA systems was the beginning of flow-through optosensing. As a result, a brief summary of the use of the solid support microbeads is mandatory. In 1976 K. Yoshimura et al. proposed the combined use of an active solid support to preconcentrate the analyte with the direct measurement of the light absorption of the species of interest sorbed on the solid phase [8], being this methodology called solid phase spectroscopy (SPS). Since then, a lot of attention has been paid to this methodology and other spectroscopic detection techniques have been implemented.

The two most remarkable analytical features of this methodology are its sensitivity and selectivity, due to the separation of the analyte from the matrix and its preconcentration on the support microbeads, that is, in the zone itself where it will be measured.

A few years later, solid phase retention was implemented with on-line spectroscopic detection, originating Flow Injection-Solid Phase Spectroscopy (FI-SPS) methodology. In this way, the advantages of SPS (sensitivity and selectivity) were added to those

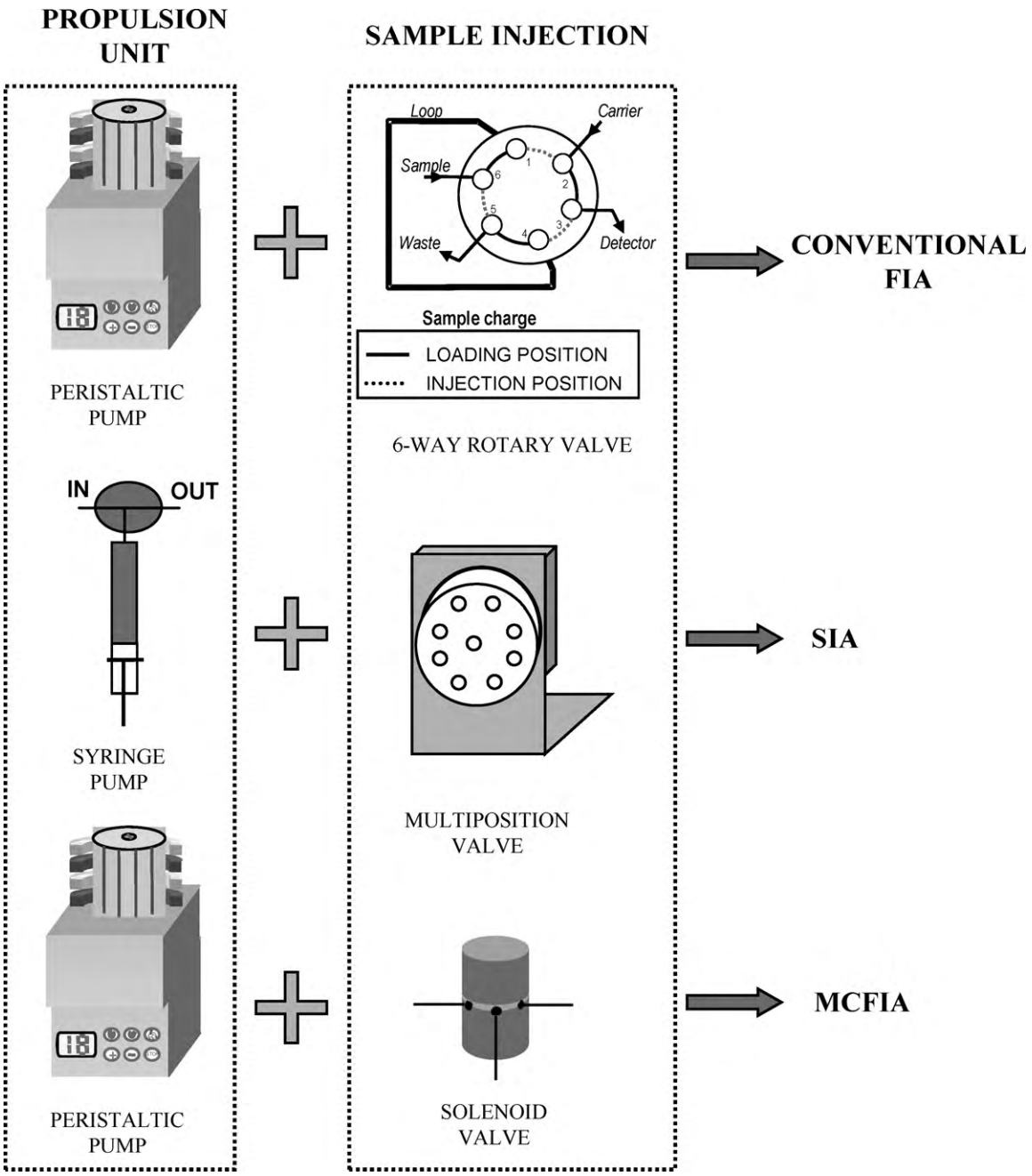

Fig. 1. Scheme of flow methodologies employed in flow-through optosensing applied to clinical analysis.

intrinsic ones from FIA (rapidity, commodity, automation, less consumption of reagents and solid supports, etc) [9]. In these FI-SPS systems the separation and retention of the species of interest on the solid phase takes place in the detection area itself and simultaneously with it. The microzone of the solid phase where the signal is continuously monitored is integrated in the detector and surrounded by a continuous stream flowing through it. The sample plug is inserted in this stream and the radiation directly interacts with the solid surface integrated in the detection area. This detection principle, based in the interaction of the radiation with the solid surface integrated in the detector is called flow-through optosensor. In addition to conventional FIA, SIA and MCFIA have been also coupled with SPS and applied in the pharmaceutical field.

The use of a solid support in the detection zone (flow-through cell) is the key element in flow-through optosensors. Solid supports used are packed in the flow-cell(s) of a conventional non-destructive optical detector. The analytes or their reaction products are immobilized on them temporarily for sensing, so integrating retention and detection, and sometimes reaction.

Sometimes, solid supports packing microcolumns coupled on-line to the flow injection system have also been used. The employ of these microcolumns has two main purposes: (a) the interference removal by use of a support suited to the sorption of the species to be removed and/or (b) to get a multi-determination. The multi-determination can be accomplished by retaining selectively one of the analytes in the microcolumn (placed just before the flow-cell) and eluting it later (after developing the signal of another one) with an appropriate reagent.

The most frequently used solid supports in flow-through sensors for pharmaceutical analysis can be divided into three groups: (a) ion-exchanging polymers (Dowex styrene polymers or Sephadex

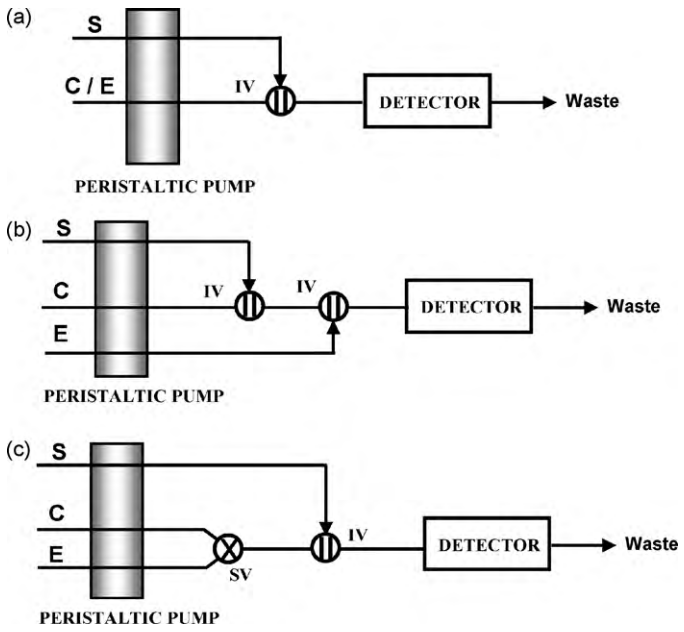

Fig. 2. Different approaches in order to regenerate the sensing zone. C: carrier; S: sample; E: eluent; IV: injection valve and SV: selection valve.

dextran polymers); (b) non-ionic polymeric adsorbents or neutral resins (Amberlite XAD) and (c) non-polar sorbents (C_{18} silica gel).

The sol–gel technology has also been introduced in the design of flow-through optosensors. It provides a simple means to incorporate, at low temperature, organic and biological recognizing elements in a stable inert support. Typically, a sol is first formed by mixing a liquid alcoxide precursor, water, a co-solvent (usually ethanol or methanol) and a catalyst (acid or base) at room temperature. This way, a porous gel network is obtained through continuous monomer hydrolysis and condensation reactions. Afterwards, gel aging and drying can be conducted in order to obtain densified solid matrices. During the hydrolysis steps, condensation or aging, the recognizing elements can be added and become entrapped in the support net, remaining sterically accessible to small analytes that diffuse into the pore network [10].

Finally, it is also worthy mentioning the employment of molecular imprinted polymers (MIPs) in recent articles [11–13]. MIPs combine highly selective molecular recognition properties with other characteristics such as physical robustness and good thermal, chemical and mechanical stability. For the preparation of MIPs, the analyte is used as template and the monomers employed form a complex with this template through covalent or non-covalent interactions. After polymerization, the template is removed and the binding sites exposed are complementary to the template in size, shape and position of the functional groups. This way, the solid support allows specific recognition sites to the target compound.

In flow-through optosensing, the solid support microbeads have to be regenerated or completely renewed after each sample determination, in order to prepare the sensing zone for the next analysis. The two main approaches employed for the regeneration have been traditionally:

(a) The carrier solution itself acts as the eluting agent. The regeneration process starts just as the tail end of the sample plug reaches the solid support in the flow-cell. In this case, a transitory signal will be developed in the detector. This is the simplest procedure to regenerate the solid sensing zone, and in turn, it allows high sampling frequencies (Fig. 2a).
(b) By using an eluting solution, which is transported to the active solid zone after the maximum signal. This approach is used when the retention of the analyte on the solid microbeads is stronger and the carrier solution itself is not enough for the regeneration. This step can be performed via an injection valve (using a definite loop) (Fig. 2b) or a selection valve (Fig. 2c). It offers a lower sampling frequency although it shows a higher sensitivity because the carrier solution does not elute the analyte from the microzone. Also, it shortens the lifetime of the sensor when an ion-exchanger is used as solid support, due to the successive swelling and compactation of the resin beads.

When the regeneration procedure is very difficult, the complete renewal of the sensing zone is performed as alternative; this is achieved by injection, transportation and trapping of a defined volume of fresh beads to the flow-cell before each analyte injection, being the beads discarded to the waste once the measurement is completed [14,15]. This is called Bead Injection Analysis (BI) and a typical manifold is shown in Fig. 3.

A chromatography glass column is used in order to obtain a homogeneous aqueous suspension of the microbeads by purging air gently through it. The procedure for each analysis consists on the introduction of an exact volume of the bead suspension via the injection valve V1. Once the beads are trapped in the flow-cell and the baseline established, the sample is injected by means of valve V2 and the analytical signal obtained on the solid support. Finally, flow direction is reversed by means of the second peristaltic pump and the beads discarded using the selection valve SV. This manifold was used for the determination of cobalt in pharmaceuticals, employing a chromogenic reagent previously loaded on the solid beads [16].

4. Detection techniques and flow-through cells

4.1. Detection techniques

The spectroscopic detection techniques employed in flow-through optosensing can be divided into three groups: (a) UV–visible spectroscopy; (b) luminescence and (c) vibrational spectroscopy.

(a) *UV–visible spectroscopy*. It has been traditionally the most frequently used detection technique in analytical laboratories. Its main handicap, the overlapping of the UV absorbance spectra from the measuring species and other accompanying compounds also present in the sample, can be minimized using flow-through sensors, as the active solid support strongly enhances selectivity excluding from the detection area all those species that cannot be retained in the experimental conditions.
(b) *Luminescence*. The techniques employed up to date in flow-through optosensing have been fluorescence, phosphorescence, lanthanide-sensitized luminescence and chemiluminescence, being the first one the most used up to date.

The most important analytical feature of fluorimetry is its high selectivity, which is accompanied by low detection limits. The added economy and simplicity, and the relative flexibility of fluorimetry, have turned it into the officially recommended choice for determining the purity of many drugs in pharmaceuticals.

In phosphorimetry, a common aspect in fluid solution is the need for some form of molecular immobilization, protection, or both, to minimize non-radiative decay of luminophores, collisions with solvent, or the possibility of photochemical reaction. Room-temperature phosphorescence in fluid solutions can be observed in the presence of micelles, heavy atoms, and nitrogen to effect deoxygenation. As it can be seen, there is a need to provide a protective, ordered medium to minimize self-quenching

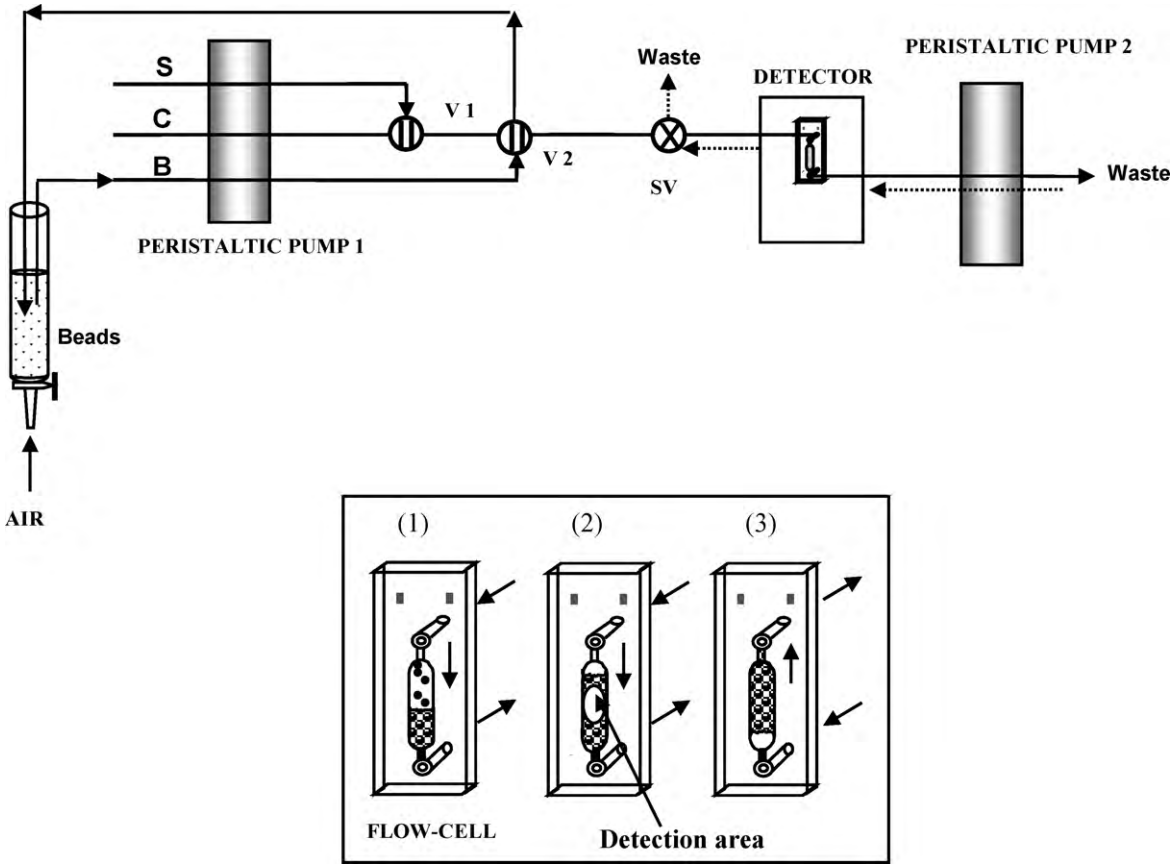

Fig. 3. Upper: BI manifold, where the dotted line in peristaltic pump 2 shows the reversal of the flow to discard the beads after each determination. Bottom: (1) the flow-cell is filled; (2) the signal is monitored; (3) the beads are discarded.

and to organize reactants on a molecular level, and to increase the proximity of heavy atoms and analyte. As a result, the use of a solid support with this purpose has lead to the development of phosphorescence flow-through sensors [17].

The analytical interest of chemiluminescence (CL) arises from the ability to produce fluorescent molecules with no prior irradiation, thereby avoiding various problems derived from light scatter, unselective excitation or light source instability. A short number of pharmaceuticals have been determined by using CL sensors [11,18–21].

Finally, lanthanide-sensitized luminescence (LSL), although an interesting detection technique due to its particular features, has been scarcely described in flow-through optosensing. In lanthanide ions complexes, the energy absorbed by the organic chromophore at its characteristic excitation wavelength is transferred to a triplet state of the molecule and then intramolecularly transferred to a resonance level of the lanthanide ion, which finally emits luminescence at its particular emission wavelength. The use of the solid phase with this detection technique offers the additional advantage of avoiding the use of enhancers or surfactants, normally used in solution lanthanide-sensitized luminescence, therefore minimizing costs and making the methodology environmentally-friendly. In LSL optosensing, both europium and terbium ions have been used [22,23].

(c) *Vibrational spectroscopy*. Although most of the sensors developed employing UV/vis absorption or luminescence detection represents very sensitive methods, they lack the great advantage of structural information. Vibrational spectroscopic detection overcomes this drawback by providing molecule-specific information. As a result, Raman spectroscopy has also been used in flow-through optosensing applied to pharmaceutical analysis [24].

4.2. Flow-through cells

Two main aims must be pursued when a suitable cell for the design of a flow-through sensor is being selected: (1) the concentration of the monitored product on the support in an area as small as possible of it and (2) the incident light beam must be focused to this area without loss of light to the surrounding zone. The best results are provided by cells with short path lengths (1–1.5 mm), which ensure compatibility between the system and the detector and prevent the species from lying outside the irradiated area.

The most appropriate commercial flow-through cell for spectrophotometric measurements in UV region is the Hellma 138-QS cell (1 mm light path, 50 μl inner volume). The cell is blocked in the outlet with glass wool to prevent particle displacement by the carrier stream and the inlet is kept free. The level of the packing material into the cell should be the necessary one to fill it up to a sufficient height, thus permitting the light beam to pass completely through the solid layer. Higher levels would imply that the support zone where the species of interest is sorbed would fall outside the detection area and so, a lower and wider signal would be obtained; with lower levels, the light beam would pass through the solution completely or partially and, consequently, a decrease in the signal would be obtained. Therefore, the top of the resin is kept as close as possible to the light beam, this latter being completely covered by the resin. The mentioned flow-cell was also used when Fourier transform Raman detection was employed [24].

In the case of chemiluminescence measurements, the Hellma 138-QS cell was the first commercial flow-cell employed in this

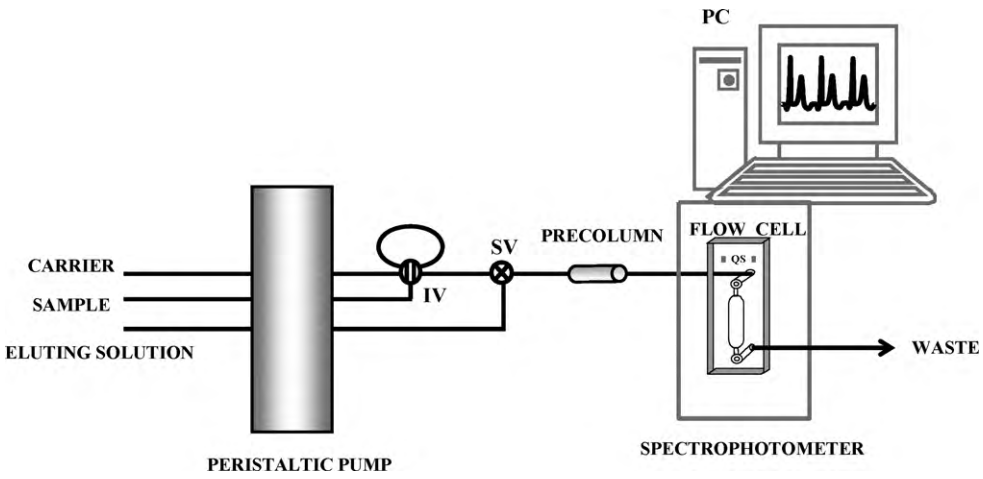

Fig. 4. Manifold employed for the separation of two analytes in a conventional FIA optosensor using a precolumn placed before the flow-cell. IV and SV stand for injection valve and selection valve, respectively.

kind of optosensors. However, the Hellma 137-QS (1 mm light path, 260 μl inner volume) was tested in a recent paper and a high increase in sensitivity was observed when compared to the first one. Finally, it is worthy mentioning that a simple glass column or methacrylate modules in different shapes can be used to retain the solid microbeads too.

For fluorescence, phosphorescence and LSL measurements, the Hellma Model 176.052-QS flow-through cell (25 μl inner volume) with a light path of 1.5 mm is usually employed. In this case, in order to secure that the species of interest retained by the packing solid material is in the light path, the solid support level is maintained just some millimeters beyond the cell window. It is necessary to take into account that, when an eluting solution other that carrier one is used to desorbs the species of interest from the solid support, ion-exchange resins suffer alternately swelling and compaction, so altering the level of the support in the cell and the baseline. This is due to the different chemical nature and concentration of the carrier and the eluting solutions. Hence, in this case, and for preventing compactions lowering the support level below the light beam, the flow-cell must be filled up passing the eluting solution through it. In every case, it is necessary to condition the solid support by passing the carrier solution through it for a few minutes.

5. Applications to pharmaceutical and clinical analysis

The applications of flow-through optosensing in these research fields have been divided according to the flow methodology employed.

5.1. Conventional FIA applications

The applications of conventional FIA optosensors with clinical or pharmaceutical applications are detailed in Table 1.

Most of the developed sensors by using this methodology are based on the direct measurement of the intrinsic UV absorbance or native fluorescence of the analyte. Some examples include the spectrophotometric determination of soluble vitamins, such as ascorbic acid or thiamine [25,26] in pharmaceutical preparations, or the monoparameter fluorescence optosensors for determining the drugs amiloride [27] and triamterene [28] in pharmaceuticals and biological fluids.

However, when the targeted species does not present intrinsic UV absorbance, a chemical reaction can be used previously to the spectroscopy measurements. The determination of ascorbic acid based on the decrease of absorbance obtained when Prussian Blue was reduced by the analyte [29] or the quantitation of promethazine and trifluoperazine after oxidation by Fe(III) and posterior complexation between Fe(II) and ferrozine [15] are two examples of this approach. In both cases, BI methodology was implemented in the system for solid support renewal.

Other approach for determining analytes that do not present native fluorescence is the so-called photochemically induced fluorescence (PIF). It consists on the generation of fluorophores from non-fluorescent analytes by on-line UV irradiation. The implementation of PIF in flow-through optosensing was described in 2005 for the determination of thiamine in pharmaceuticals and human biological fluids [30]. Other recent methods have been applied to the determination of several drugs, such as reserpine or flufenamic acid [31,32].

When more than one analyte is determined in the same sample insertion, the analytes have to be separated previously to the analytical measurements. The separation of the analytes has been usually performed using a mini-column filled with the same solid support used in the flow-through cell. When the sample solution is inserted in the system, one analyte goes through the mini-column while the other analyte/s stay retained on the solid microbeads in the column; once the first analyte signal is recorded, the retained species can be selectively eluted using appropriate eluting solutions and pumped towards the flow-cell, developing their transitory analytical signals on the solid support. This approach has been used, for instance, for determining mixtures of paracetamol/caffeine and sulfamethoxazole/trimethoprim [33,34]. A manifold showing this kind of separation is depicted in Fig. 4.

Chemiluminescence has been used as detection technique using two main approaches. The most commonly employed one has been to place the solid support inside the flow-cell, which is situated in front of the window of the photomultiplier tube (PMT), while the latter method consists on immobilizing the reagents on a solid support placed just before the cell, releasing them in the appropriate moment to obtain the CL reaction with the analyte. When the solid support is placed inside the flow-cell, two options are available: (a) both reagents and analyte solutions can be delivered towards the cell and (b) the reagents can be previously immobilized on the solid support and only the sample solution is inserted for each determination. One example of the first case is depicted in Fig. 5a for the determination of salbutamol [11]. This method is based on the sensitization produced by the analyte on the CL reaction between luminol and ferricyanide potassium. A molecular imprinted polymer (MIP) was employed as solid support and all solutions pumped towards the cell for each sample determi-

Table 1
Conventional FIA applications.

Analyte	Solid support	Matrix	Pre-treatment operation	DL ($\mu g\,ml^{-1}$)	Detection technique	Ref.
Acetaminophen	C_{18} silica gel	Pharmaceuticals	Separation on a mini-column	0.5	Spectrophotometry	[52]
Acetylsalicylic acid				0.8		
Caffeine				0.3		
Amiloride	SPC-25	Pharmaceuticals; serum		0.00033	Fluorescence	[27]
Ascorbic acid	SPC-25	Pharmaceuticals	Separation on solid support	7	Spectrophotometry	[25]
Acetylsalicylic acid				100		
Thiamine				0.75		
Ascorbic acid	QAE A-25	Pharmaceuticals	Two different flow-through cells	0.36	Spectrophotometry	[26]
Thiamine	SPC-25			0.14		
Caffeine	C_{18} silica gel	Pharmaceuticals	Separation on a mini-column	0.56	Spectrophotometry	[34]
Paracetamol				0.75		
Ciprofloxacin	Amberlite IRC-50S	Pharmaceuticals	Complexation with europium	0.033	LSL	[22]
Nafronyl	Silica gel Davisil	Pharmaceuticals; urine		0.021	Fluorescence	[53]
Naproxen	Amberlite XAD 7			0.013		
Ofloxacin	CR-1211 sponge	Pharmaceuticals	Oxidation with PbO_2	0.078	Chemiluminescence	[18]
Pipemidic acid	CR-1211 sponge	Pharmaceuticals	Oxidation with $NaBiO_3$	0.062	Chemiluminescence	[19]
Sulfonamides	QAE A-25	Pharmaceuticals; urine		0.1	Spectrophotometry	[54]
Triamterene	SPC-25	Pharmaceuticals; serum		0.00017	Fluorescence	[28]
Ascorbic acid	QAE A-25	Pharmaceuticals	Complexation with Ferrozine; BI	0.02	Spectrophotometry	[14]
Iron				0.003		
Cobalt	Dowex 50 W	Pharmaceuticals	Complexation with 1-(2-pyridylazo)-2-naphthol; BÏ	0.019	Spectrophotometry	[16]
Caffeine	C $_{18}$ silica gel	Pharmaceuticals	Separation on a mini-column	0.65	Spectrophotometry	[55]
Paracetamol				7.5		
Propyphenazone				1.9		
Propanolol	Amberlite XAD-7	Pharmaceuticals; urine		0.001	Fluorescence	[56]
Reserpine	Amberlyst A-27	Pharmaceuticals; urine	Oxidation between luminol and periodate	0.0003	Chemiluminescence	[35]
Sulfamethoxazole	SPC-25	Pharmaceuticals	Separation on a mini-column	9.5	Spectrophotometry	[33]
Trimethoprim				0.6		
Analgin	CR-1211 sponge	Pharmaceuticals	Oxidation with MnO_2	27	Chemiluminescence	[57]
Ascorbic acid	QAE A-25	Pharmaceuticals	Reduction of Prussian blue	0.08	Spectrophotometry	[29]
Ciprofloxacin	SPC-25	Pharmaceuticals		0.035	Spectrophotometry	[58]
Diphenhydramine	G-15	Pharmaceuticals		0.019	Fluorescence	[59]
Naphazoline	Amberlite XAD-7	Pharmaceuticals	Use of KI	0.0094	Phosphorescence	[17]
Promethazine	QAE A-25	Pharmaceuticals	Oxidation by Fe (III), followed by	0.09	Spectrophotometry	[15]
Trifluoperazine			Fe (II) and ferrozine complexation. BI	0.14		
Quinine	SPC-25	Pharmaceuticals		0.0001	Fluorescence	[60]
Quinidine				0.0002		
Caffeine	C_{18} silica gel	Pharmaceuticals		0.09	Spectrophotometry	[61]
Theophylline				0.09		
Copper	QAE A-25	Pharmaceuticals	Chromogenic reagent zincon. BI	0.029	Spectrophotometry	[62]
Zinc				0.04		
Naphazoline	Amberlite XAD-7	Pharmaceuticals		0.0026	Fluorescence	[63]
Norfloxacin	SPC-25	Urine; serum	Complexation with Tb(III)	0.0015	LSL	[23]
Salbutamol	MIP	Urine	Luminol and potassium ferricyanide reaction	0.016	Chemiluminescence	[11]
Sulfamethoxazole	QAE A-25	Pharmaceuticals		100	Raman spectroscopy	[24]
Sulfathiazole				100		
Thiamine	C_{18} silica gel	Pharmaceuticals; serum; urine	UV irradiation	2.8×10^{-5}	Fluorescence	[30]
Vanadium	QAE A-25	Serum; urine	Complexation with Alizarin Red S	0.00045	Fluorescence	[64]
Reserpine	C_{18} silica gel	Pharmaceuticals; serum; urine	UV irradiation	5×10^{-5}	Fluorescence	[31]
Sulfanilamide	QAE A-25	Pharmaceuticals; urine	UV irradiation	0.0029	Fluorescence	[65]
Sulfamethoxazole				0.0081		
Sulfathiazole				0.0057		
Digoxin	MIP	Serum		1.7×10^{-5}	Fluorescence	[66]
Norfloxacin	AG 1-X8	Pharmaceuticals; serum	$KMnO_4$ and Na_2SO_4 CL reaction followed by LSL	0.0028	LSL	[36]
p-Aminobenzoic acid	QAE A-25	Pharmaceuticals	Complexation with Tb(III)	0.06	LSL	[37]
Fenfluramine	MIP	Pharmaceuticals	Oxidation with $KMnO_4$	1	Chemiluminescence	[12]

nation. Another alternative would be to retain the oxidant on the solid support previously to the insertion of the analyte, which is the only solution pumped towards the flow-cell, where the reaction takes place. This approach has been used for the determination of ofloxacin using PbO_2 retained on a sponge rubber [18] or for the quantitation of pipemidic acid based on its sensitizing effect on the CL oxidation of sulphite by sodium bismuthate, which was previously immobilized on the solid support inside the flow-cell [19]. The second approach was applied to the determination of reserpine in pharmaceutical preparations and human urine samples [35]. The CL reagents, luminol and periodate, used in this sensor, were both immobilized on anion-exchange resin. Through injection of 100 μl eluent, the reagents on the anion-exchange resin column were eluted and in the presence of reserpine, the CL intensity was decreased, by which reserpine could be sensed. The sensor showed stability without replacing the resin microbeads for at least 80 h. The manifold employed in this method is depicted in Fig. 5b.

One paper has been published comparing different phosphorescence methodologies for determining naphazoline in pharmaceuticals [17]. One of the possibilities consisted on an optical sensor, employing iodide as heavy atom and Amberlite XAD-7 as the solid support. The sensitivity and repeatability obtained with the optosensor was similar to the ones observed in solution.

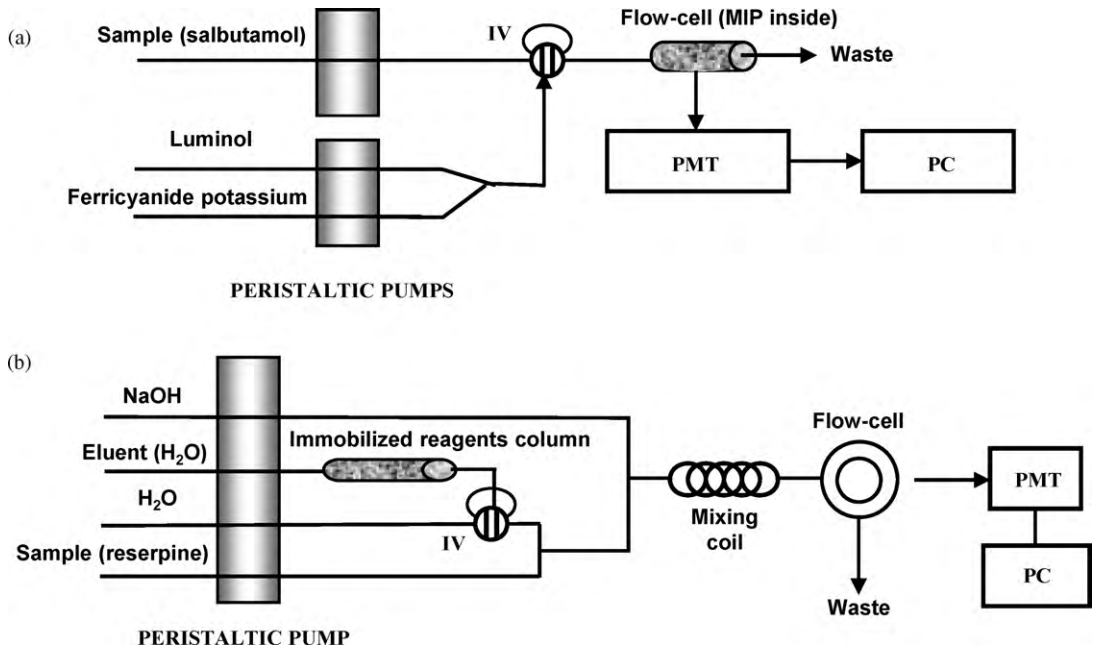

Fig. 5. Manifolds employed in CL flow-through optosensing. (a) Reagents and analyte solutions delivered towards the cell and (b) reagents previously immobilized on the solid support.

LSL has been used in several optosensors, employing Eu (III) or Tb (III) as the lanthanide ion. The determination of ciprofloxacin was achieved by means of europium ion [22] while terbium was used for determining norfloxacin [23,36] and p-aminobenzoic acid [37]. In all cases, high sensitivity and selectivity was observed due not only to the solid support, but also to the highly specific detection technique.

Finally, an optosensor using Fourier transform Raman spectroscopy has been recently developed for determination of sulfonamides [24]. The molecular and structural information contained in Raman spectra together with the selective retention of the species of interest on an anion-exchange sorbent made this method highly selective. The direct quantitative determination of sulfathiazole and sulfamethoxazole in the presence of other species normally encountered with these analytes was satisfactorily proved.

5.2. SIA applications

The applications of SIA optosensing to clinical and pharmaceutical analysis are shown in Table 2. In the development of these optosensors, fluorescence, TSL and CL have been employed as detection techniques. Two fluorescence monoparameters have been described for the determination of labetalol [38], measuring its native fluorescence when retained on C_{18} silica gel, and paracetamol [39] by means of a derivatization reaction performed off-line.

The quantitation of 5-aminosalicylic acid in pharmaceuticals has been carried out measuring the chemiluminescence obtained by its oxidation on a solid support using permanganate in an acidic medium [21]. In this case, a comparison between two different Hellma flow-through cells was performed, observing a high increase in sensitivity when using the highest inner volume. In addition, not only SIA was employed, but also MCFIA. As a result, a critical discussion of the parameters observed for both systems could be made, focusing on their possible application to the routine analysis of pharmaceuticals.

Two TSL optosensors were developed for the individual determination of salicylic acid and cromolyn. The former method consisted on the sorption of the analyte on an anion-exchanger solid support, followed by complexation with Tb(III) on the microbeads; it was satisfactorily applied to the determination of salicylic acid in pharmaceuticals [40]. On the other hand, the complex between cromolyn and terbium ion was formed on-line previously to its sorption on a cation-exchanger resin, Chelex-100. This last optosensor was applied to the determination of the analyte in both pharmaceutical preparations and human urine [41].

Finally, a recent paper demonstrated the versatility of SIA for the design of complex automatic optosensors [42]. This method presented a novel characteristic: the use of two different detection techniques in the same system, therefore enhancing the scope of applications. This approach allowed the determination of compounds with dissimilar spectroscopic characteristics. The manifold employed for the determination of vitamins B2, B6 and C is depicted

Table 2
SIA applications.

Analyte	Solid support	Matrix	Pre-treatment operation	DL ($\mu g\,ml^{-1}$)	Detection technique	Ref.
Labetalol	C_{18} silica gel	Pharmaceuticals; urine		0.0033	Fluorescence	[38]
Paracetamol	QAE A-25	Pharmaceuticals	Reaction with $NaNO_2$	2	Fluorescence	[39]
Ascorbic acid	QAE A-25	Pharmaceuticals	Reaction with MnO_4^- on solid support	9.1	Chemiluminescence	[42]
Pyridoxine	C_{18} silica gel		Separation on the flow-cell	0.12	Fluorescence	
Riboflavine	C_{18} silica gel		Separation on the flow-cell	0.008	Fluorescence	
Salicylic acid	QAE A-25	Pharmaceuticals	Complexation with Tb(III) on solid support	0.045	LSL	[40]
5-Aminosalicylic acid	QAE A-25	Pharmaceuticals	Reaction with MnO_4^- on solid support	0.3	Chemiluminescence	[21]
Cromolyn	Chelex-100	Pharmaceuticals; Urine	Complexation with Tb(III)	0.015	LSL	[41]

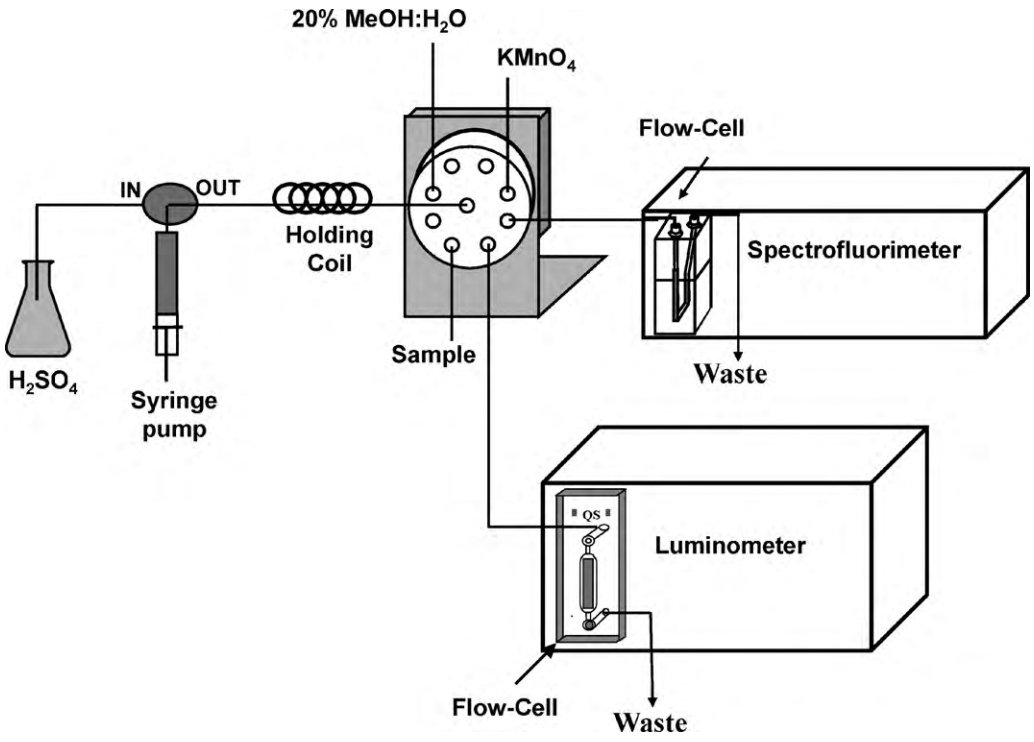

Fig. 6. Sequential injection optosensor for determining a mixture of 3 vitamins using two luminescence detectors.

in Fig. 6. By means of the SIA multivalve, the sample could be easily pumped towards the required detector, therefore allowing the use of the double-detection technique. Taking into account the native fluorescence of B6 and B2, these analytes were determined by means of a spectrofluorimeter. However, as ascorbic acid does not present native fluorescence, its reaction with permanganate in a sulphuric medium was performed and the obtained chemiluminescence measured. In every case, the determination was based on the direct measurement of the analytical signal of the respective vitamin retained on the sensing solid support placed on the respective detection area. Two different solid supports were used, a non-ionic C_{18} silica gel for the fluorescent compounds and an anionic QAE A-25 for ascorbic acid.

5.3. MCFIA applications

The applications of multicommutated optosensors to clinical analysis are detailed in Table 3.

Table 3
MCFIA applications.

Analyte	Solid support	Matrix	Pre-treatment operation	DL ($\mu g\,ml^{-1}$)	Detection technique	Ref.
Bismuth	Sol–gel	Pharmaceuticals	Complexation with xylenol orange	0.007	Spectrophotometry	[10]
Copper	Sol–gel	Urine	Complexation with 4-(2-pyridylazo)resorcionol	0.003	Spectrophotometry	[43]
Zinc	Sol–gel	Pharmaceuticals	Complexation with 4-(2-pyridylazo)resorcionol	0.002	Spectrophotometry	[44]
Acetazolamide	Sol–gel	Pharmaceuticals	Enzimatic measurements	44.5	Spectrophotometry	[45]
Furosemide	SPC-25	Pharmaceuticals; urine; serum	Separation on precolumn	0.015	Fluorescence	[48]
Triamterene				0.0001		
Pipemidic acid	SPC-25	Pharmaceuticals; urine; serum	Complexation with Tb(III)	1.79×10^{-7}	LSL	[67]
Salicylamide	C_{18} silica gel	Pharmaceuticals	Separation on precolumn	0.33	Spectrophotometry	[47]
Caffeine				0.15		
Piroxicam	C_{18} silica gel	Pharmaceuticals	Separation on precolumn	0.27	Spectrophotometry	[46]
Pirydoxina				1.2		
Pyridoxine	C_{18} silica gel	Pharmaceuticals	Separation on the flow-cell	0.045	Fluorescence	[51]
Riboflavine				0.003		
Salicylic acid	QAE A-25	Pharmaceuticals	Reaction with MnO_4^- on solid support	0.3	Chemiluminescence	[20]
Caffeine	C_{18} silica gel	Pharmaceuticals	Separation on precolumn	0.21	Spectrophotometry	[49]
Salicylamide				0.61		
Propyphenazone				0.3		
Flufenamic acid	C_{18} silica gel	Pharmaceuticals; serum; urine	UV irradiation	0.00015	Fluorescence	[32]
Naproxen	C_{18} silica gel	Pharmaceuticals; urine; blood	Separation with solid support in flow-cell	0.0003	Fluorescence	[50]
Salicylic acid				0.0013		
5-Aminosalicylic acid	QAE A-25	Pharmaceuticals	Reaction with MnO_4^- on solid support	0.3	Chemiluminescence	[21]

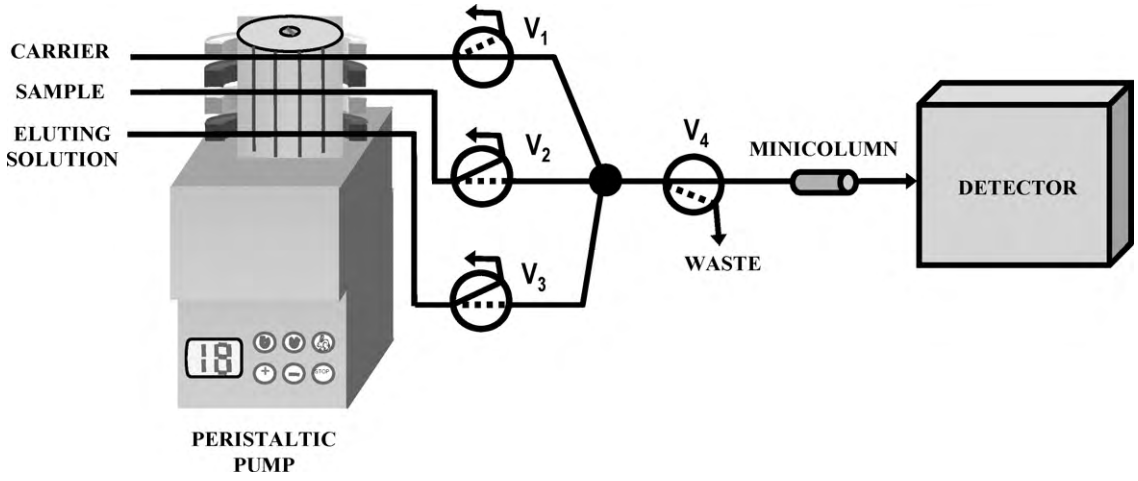

Fig. 7. MCFIA optosensor for the separation of a mixture of two analytes. V_i stands for solenoid valve.

The early flow-through optosensors using MCFIA methodology were described by Jerónimo et al. in 2004 and were designed employing the sol–gel technology. Three of them were applied to the determination of inorganic elements in pharmaceuticals and biological fluids. In these sensors, a colorimetric organic compound was entrapped in the sol–gel inert support in order to form a complex with the analyte when arriving to the flow-cell. This way, copper, bismuth and zinc were satisfactorily determined [10,43,44]. An optical biosensor was also described for the determination of acetazolamide. In this case, carbonic anhydrase was immobilized on the support and the analyte was determined by enzyme inhibition measurements [45].

The optosensors developed employing MCFIA systems have dealt not only with the determination of one analyte, but also for determining more than one. In the latter case, the separation of the analytes can be made using a mini-column filled with the same solid support used in the flow-through cell or increasing the amount of solid support in the same flow-through cell (when the retention/desorption kinetic of the analytes on the solid support is very different, the separation can be performed by just placing an extra amount of microbeads in the flow-though cell, instead of using a mini-column). Next, both cases are commented.

In Fig. 7, the typical manifold employing a mini-column for the separation of two analytes is depicted. The sample is introduced by activating valves V_1 and V_2; one of the analytes is retained on the solid support of the mini-column while the other one passes through this and is retained on the solid support in the flow-cell. This first analyte develops its analytical signal on the sensing zone and is eluted from the microbeads by the carrier itself. After that, valves V_1 and V_3 are activated and the second analyte is eluted by an appropriate eluting solution from the mini-column and propelled towards the sensing zone, where it develops its corresponding analytical signal. Valve V_4 is used in the final cleaning step. This system has been used for the spectrophotometric determination of pirydoxine/piroxicam [46] and salicylamide/caffeine [47] in pharmaceutical preparations, as long as the fluorimetric quantitation of furosemide and triamterene in pharmaceuticals and biological fluids [48]. A triparameter optosensor has been also developed for determining salicylamide, caffeine and propyphenazone in pharmaceuticals. In this case, the separation was also performed using a mini-column, but two eluting solutions were required [49].

When the separation of the analytes is performed in the own flow-cell, an additional solid support placed just above the detection area in the same cell is necessary. Employing the exact configuration of valves, this manifold was used for the fluorimetric determination of naproxen/salicylic acid [50] in biological fluids and pyridoxine/riboflavin [51] in pharmaceuticals.

Two multicommutated optosensors have been recently developed for the chemiluminometric determination of salicylic acid and 5-aminosalicylic acid [20,21]. In both cases, the chemiluminescence reaction consists on the direct oxidation of the analyte by permanganate ion in acidic medium. Permanganate solution is first inserted in the flow system and the ions retained on an anion-exchanger solid support; after that, the sample is inserted in the system and the reaction takes place on the same microbeads, therefore obtaining the analytical signal.

6. Comparison of flow methodologies

All the flow methodologies employed in the development of flow-through optosensors have favourable intrinsic characteristics, as previously stated. However, each one presents specific advantages when compared to each other. We will make a comparison between these methods in term of the degree of automation, repeatability, sensitivity and sample frequency.

The introduction of multicommutation and SIA has provided additional automation when compared to FIA, due to the complete absence of human intervention during each analysis. In FIA, an operator has to switch manually the rotary valves in order to inject the sample or reagents solutions. In recent MCFIA and SIA optosensors, the flow of solutions is automatically controlled by appropriate software and the consumption of sample and reagents is lower, making these methods more suitable for routine analysis.

The repeatability of SIA and MCFIA methods is theoretically higher when compared to FIA ones due to the higher degree of automation. However, a comparison of the R.S.D. (%) reported in recent published papers show similar results in all cases. This is due to the use of the solid support, which compensates the theoretically small difference that could be found when working in homogeneous solutions.

The sensitivity of MCFIA and conventional FIA methods is slightly better when compared to SIA. This is due to the dilution of the sample in the SIA system when all solutions have to be aspirated previously to be pumped towards the detection area. This effect has been proved in a previous paper comparing the use of MCFIA and SIA for the same analysis [21]. For the same reasons of the aspiration/pumping process in SIA, the sample frequency in these methods is lower when compared to MCFIA or classical FIA.

In general, the incorporation of multicommutation and SIA to flow-through optosensing has provided the laboratories with

favourable tools in order to develop automatic, rapid and environmental-friendly methods for routine analysis.

7. Conclusions

Advances in medicine raised an increasing demand for control analyses and posed various challenges to analytical chemists such as the need to develop new methods exhibiting as much sensitivity, selectivity, easy automation, low cost of equipment, simplicity and environmental friendliness as possible. This article reviews the recent advances in flow-through optosensing applied to clinical and pharmaceutical analysis. The automatic flow methodologies applied to SPS i.e. FIA, SIA and MCFIA, have proved their applicability in this type of analysis, therefore demonstrating innovative alternatives to classical flow techniques. The use of SIA and MCFIA has introduced a great number of advantages, mainly a dramatic decrease of the needed sample and reagents volumes and complete automation. In addition, new detection techniques such as Raman spectroscopy or PIF have been used in recent years, providing the analyst with a wider range of options for the design new analytical methods.

In the future, the implementation of SPS with other flow methodologies such as multipumping or lab-on valve would highly improve the manifolds, providing further miniaturization of the systems and higher versatility in the design of automatic methods for routine analysis.

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