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Determination of asulam by fast stopped-flow chemiluminescence inhibition of luminol/peroxidase

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ABSTRACT

An efficient, sensitive and fast stopped-flow method has been developed to determine asulam in water, based on its inhibition effect on the horseradish peroxidase–luminol–hydrogen peroxide chemiluminescence reaction, (HRP–luminol–H_2O_2). Ultra fast data acquisition (0.20 s) facilitates excellent selectivity because no interferences from concomitants in the matrix act in such short time scale. The precision as repeatability (expressed as relative standard deviation, $n = 10$) was 0.4% at a 40 pM level. The detection limit was 1.5 pM (0.35 ng/L) and 7.15 pM in pure and raw water, respectively. The calibration data over the range 5–60 pM present a correlation coefficient of $r = 0.9993$. The proposed method has been applied to determine asulam in water samples by using solid-phase extraction (SPE). Mean recovery value was 98.1 ± 2% at 50 pM level.

1. Introduction

Asulam (methyl 4-aminobenzenesulfanilyl carbamate) is a carbamate pesticide with a widely broad spectrum of biological activity, acts by stopping the cell division and growth of plant tissues, can accumulate and remain in environment. Due to its good solubility in water, creates a compromise to develop suitable analytical methods [1,2]. In literature, a great variety of analytical techniques are employed to quantified asulam at trace level. Currently, gas or liquid chromatography is the most used method for analyzing pesticides [3], and solid-phase extraction (SPE) is often performed for concentrating and purifying analytes in the samples, prior analysis [4–6]. Analytical determination by means of synchronous fluorescence with derivatization with fluorescamine [7], or based on its native fluorescence with a flow injection (FI) analysis system, has been used [8,9].

Chemiluminescence (CL) is a luminescence technique showing as main advantages its high sensitivity, easy of use and simple instrumentation, being actively applied for the detection of small amounts of chemical species at ultra-trace levels. Considering the kinetic characteristics of this technique, anyone of the reaction components, including CL substrate, oxidant, catalyst, cofactor, sensitizer, enhancer and inhibitor can be made rate limiting and hence be determined [10]. The oxidation is usually conducted in a basic solution in the presence of an oxidant: hydrogen peroxide [11], hypochlorite [12], iodine [13], or oxygen [14], and catalysts: peroxidases such as horseradish peroxidase (HRP) [15,16], or lactoperoxidase [17], and metals ions [18]. Among them, HRP–luminol–H_2O_2 system is most popular and has been employed in many assays [19,20]; moreover, this system has been employed to quantify analytes who act as enhancers [21,22] or inhibitors [23]. Particularly, in the determination of carbamates as analytes, only few contributions have been found based on the CL of $Ru(bpy)_3^{3+}$ reaction [24], and on the peroxyoxalate CL oxidation in the presence of a fluorophore [25]. This technique can be easily coupled to a flow injection manifold as detection mode or assembled to stopped-flow technique, where rapid chemical reactions are studied on the millisecond to second time scale [26].

In this study, we have found that the pesticide asulam produces an inhibition on the CL emission from the HRP–luminol–H_2O_2 system. Under the optimum experimental conditions, the very fast CL emission is proportional to the concentration of asulam, the whole transient signal can be recorded in about 200 ms. The main features of this technique are its ability to mix the sample and reagent solutions automatically, the possibility of making experiments shortly after mixing, a high overall precision, the minimization of potential interferences, and its suitability for fast and slow reactions. Based on these findings, a simple and fast new direct stopped-flow CL method has been developed for the determination of asulam in water by using solid-phase extraction.

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2. Experimental

2.1. Reagents and solutions

Ultrapure water (Millipore 60 system, Bedford, MA, USA) was used for the preparation of all solutions, except asulam which was prepared in acetone. Luminol (5-amino-2,3-dihydrophthalazine-1,4-dione), horseradish peroxidase, type VI-A (16 U/mg) and Tris–HCl (99–99.5%) were purchased from Sigma (St. Louis, USA). Hydrogen peroxide (H_2O_2) 30% (p/v) by Merck (Darmstadt, Germany). Asulam (methyl[(4-aminophenyl)sulfonyl]carbamate) (98.1%), MCPA (4-chloro-*o*-tolyloxyacetic acid), 2,4,5-T (2,4,5-trichlorophenoxyacetic acid), amitrole (3-amino-1,2,4-triazole), and atrazine (2-chloro-4-(ethylamine)-6-(isopropylamine)-*s*-triazine) were purchased from Riedel de Häen, Seelze, Germany and employed without further purification. Diclorprop (2-(2,4-dichlorophenoxy) propanoic acid) and metamidofos (*O*,*S*-dimethyl phosphoramidothioate) was provided by Dr. Ehrenstoffer Laboratories (Augsburg, Germany).

The stock solution of luminol (2 mM) was prepared by dissolving 35.44 g of solid in a 0.1 M of Tris–HCl buffer solution (pH 8.4); this solution is stable when stored in the dark. 13.68 U/mg of lyophilized HRP was prepared in water by dissolving 1.3 mg of the parent HRP in 25 mL. H_2O_2 (1 M) was prepared in water by dissolving 752 μL in a volume of 25 mL. The concentration of H_2O_2 was determined daily by measuring the UV absorbance at 240 nm using $\varepsilon_{240} = 39.4\ cm^{-1}\ M^{-1}$ [27]. Asulam (1 mM) stock solutions were prepared by dissolving in acetone 11.51 mg to a final volume of 50 mL.

2.2. Apparatus

A Perkin-Elmer LS-50 (Beaconsfield, UK) luminescence spectrometer with the light source switched-off was used. The apparatus was set in the phosphorescence mode with 0.00 ms delay time and 60 ms gate time. The slit-width of the emission monochromator was set at 20 nm with λ = 425 nm and photomultiplier voltage set manually to 700 V. The samples were placed in a quartz cuvette continuously stirred with a magnetic stirrer. The chemiluminescent reaction was triggered by injecting HRP solution with a syringe, through a septum.

The study of the CL emission at short times was carried out in an SLM-Aminco 48000s fluorometer equipped with a MilliFlow stopped-flow reactor (Urbana, IL), with the light source turned off and no optical filter before the photomultiplier. The stopped-flow reactor permits observation of the reaction velocity of the reactants which are forced through a mixing chamber and into an observation cell. The two 5 mL driving syringes were filled with the reactants and whose positions are simultaneously driven by a pressurized nitrogen-operated plunger. After leaving the observation cell, the mixed reactants advance the piston of a stopping syringe, which is brought to a "dead" stop against the tip of a micrometer. Just prior to stopping, a switch is closed, thereby supplying a triggering signal which opens the electronic data acquisition. The cell volume was 32 μL. Equal volumes of the two reagent solutions were introduced into the cell when a pressure of 4.5 bar was applied on the two supply syringes. The dead time was 1.0 ms, flow velocity 20 mL/s, and mixing efficiency was >98%. The intensity (in V) was collected throughout the reaction at a rate of 10 ms per point with a photomultiplier gain of 10 and a voltage of 900 V.

2.3. Methods

2.3.1. General procedure

For the study of the asulam signals at short times, a syringe of the stopped-flow apparatus was filled with HRP (0.6 U/mL) in 0.02 M buffer solution (pH 8.4) and the other with a mixture of luminol (3×10^{-5} M), H_2O_2 (2×10^{-3} M), 0.02 M buffer solution and variable volumes of asulam to obtain 5–60 pM concentrations. The initial rates (d*I*/d*t*) were measured by subtracting the signal values at 0.08 s from that at 0 s. All measurements were carried out at 25 ± 0.1 °C.

2.3.2. Procedure for the determination of asulam in water

Due to the good solubility of this pesticide in water [28], a preconcentration step is necessary to reach at trace level the limit of detection. Solid-phase extraction was performed with Lichrolut SPE RP-18 (500 mg, 6 mL) from Merck. Previously, the SPE cartridges were conditioned by passing, according to the manufacturer recommendations, 2 mL of methanol followed by 10 mL of LC-grade water through the cartridge at a flow rate of 3 mL/min. The extraction tubes were set with a Visiprep™ SPE Vacuum Manifold system purchased from Supelco. Fifty milliliters of asulam solution (10^{-8} M) were percolated through the C_{18} cartridges at a constant flow rate between 4 and 8 mL/min. After preconcentration, the extraction was eluted with 2 mL of methanol and evaporated to dryness with a gentle stream of nitrogen and reconstituted with 500 μL of water.

Equal volumes of the two reagent solutions were introduced into the cell when a pressure of 4.5 bar was applied on the two supply syringes. All measurements were carried out at 25 ± 0.1 °C.

3. Results and discussion

3.1. Optimization of the factors affecting the asulam determination at short times

The experimental conditions were optimized by means of the univariate approach. The efficiency of HRP–luminol–H_2O_2 system is highly dependent on reaction pH. Tris–HCl buffer pH has been reported previously to give a higher CL intensity with luminol as compared with other buffer solutions [19]. Therefore, in the proposed method, the effect of Tris–HCl concentration was studied in the range 0.01–0.1 M. The maximum CL inhibition with asulam was observed at a Tris–HCl concentration of 0.02 M. The effect of Tris–HCl buffer pH (0.02 M) was investigated in the range 8.2–9.5, and the maximum CL was observed at pH 8.4 (Fig. 1); therefore, Tris–HCl buffer (0.02 M and pH 8.4) was selected and used for subsequent studies. The effect of luminol concentration was studied in the range 5–150 μM, the best results were obtained

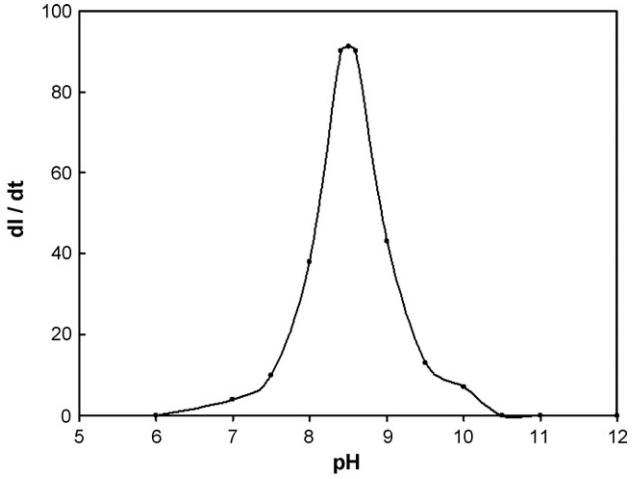

Fig. 1. Initial slope (0–80 ms) at several pH values. Experimental conditions: [Tris–HCl buffer] = 0.02 M; [HRP] = 0.6 U/mL; [luminol] = 3×10^{-5} M; H_2O_2 (2×10^{-3} M).

Table 1
Inhibition data for asulam ($n = 5$)

Asulam (pM)	Inhibition (%)	R.S.D. (%)
0	1.10	3.04
20	25.93	0.81
40	48.96	2.81
80	66.53	3.05
120	81.25	1.09
160	87.23	1.34
320	89.51	1.42
400	95.03	0.8

at 30 μM, compromises between signal reproducibility and blank signal value. Further experiments were realized using 30 μM as optimum luminol concentration. The effect of hydrogen peroxide on the determination of asulam was investigated in the range of 0.2–3 mM, and the best result was obtained with 2 mM. Same procedure was carried out with the peroxidase (HRP) concentration, reaching the 0.6 U/mL as the optimum value of analysis.

3.2. Study of the CL with asulam at short time

The preliminary experiment, carried out in the SLM-Aminco apparatus, showed the CL reaction of luminol was inhibited by asulam, at very low level of concentrations (pM). Blank signals were obtained using the luminol–H_2O_2 system by itself and catalyzed by peroxidase. In Table 1 are presented the inhibition data of this system and illustrated in Fig. 2, measured between 0 and 120 ms and were obtained the slopes of the initial rates (dI/dt) versus asulam concentration over the range 20–400 pM. The data are obtained with the aid of the following expression:

$$\%I = \left(\frac{S_b - S}{S_b}\right) \times 100$$

where I is the inhibition (in %) and S_b and S are the mean of five separate measurements of the slope of the blank and signals, respectively.

3.3. Quantitative analysis and main analytical figures of merit

The calibration data of CL inhibition versus concentration of asulam were obtained over the range 0–400 pM. According to the experimental data, the linear analytical range was established between 5 and 60 pM. A calibration graph obtained by least-squares treatment was: $\%I_{CL} = 1.19$ [asulam] + 1.93627 ($n = 5$), with a correlation coefficient of 0.9993 and R.S.D. = 0.40%. The detection limit (1.5 pM, 0.35 ng/L) was calculated as three times the standard deviation of seven blanks divided by the slope of the calibration graph. Quantification limit (10σ) was 5 pM.

Table 2
Interference study at 50 pM asulam level

Interference	Asulam:interference (w/w)	Recovery (%)
2,4,5-T	1:1	88.2
	1:0.5	95.6
Diclorprop	1:1	98.6
MCPA	1:1	98.7
Atrazine	1:1	79.2
	1:0.5	86.9
	1:0.25	88.9
Metamidofos	1:1	98.1
Amitrole	1:1	98.9

3.4. Interferences

In order to study the determination of asulam in real water samples, the effect of other pesticides that can be present in water at similar levels was studied. In Table 2 are ordered the results obtained over samples containing 50 pM asulam. The selectivity of the proposed methods against the pesticides checked is confirmed, except for atrazine that cause a severe interference (1:0.25, 88.9%).

3.5. Application

In order to assess the potential of the method described, the proposed method was applied to the recovery of asulam in tap water samples, collected from the university. After collection, samples were filtered through a cellulose membrane filter (cellulose acetate, pore size 0.45 μm, 47 mm diameter, Whatman, Maidstone, UK), to remove some possible suspended solids. Into a 50 mL calibrated flask were transferred an aliquot of 25 μL of 10^{-8} M asulam standard solution, the final concentration was of 5 pM (1.15 ng/L).

According to the procedure described above (see Section 2.3), the solid-phase extraction was applied and best recoveries were obtained when the pH of the solution was adjusted at pH 3. With this, a calibration graph was obtained from the kinetic curve (Fig. 3) at the 175 ms over the range 5–60 pM. Least-squares treatment gives $\%I_{CL} = 1.445$ [asulam] − 6.603 ($n = 5$), with a correlation coef-

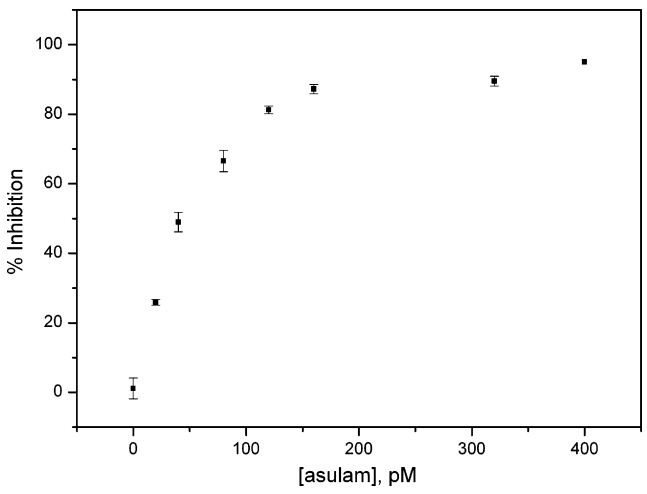

Fig. 2. % Inhibition against asulam concentration (pM). Conditions as in Fig. 1.

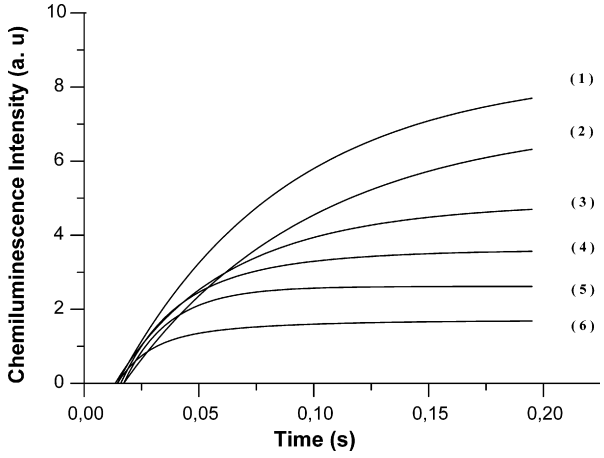

Fig. 3. Kinetic curves of the calibration graph for asulam in tap water. (1) 0 pM; (2) 20 pM; (3) 30 pM; (4) 40 pM; (5) 50 pM; (6) 60 pM. Analytical signal obtained at 175 ms.

Table 3
Comparison of the performances of the proposed method with the previously published one

Sample	DL	Recovery (%)	R.S.D. (%)	Technique
Tap water	0.35 ng/L	98	1.90	This method
Tap water	1 μg/L	86	6.50	MEKC-UV detection [29]
Tap water	0.8 μg/L	88	5.80	MEKC-electrochemical detection [29]
Tap water	0.04 ng	90–118	1.13	HPLC with derivatization [30]
Natural water	10.9 μg/L	53–98	<5.3	CE-UV detection [31]
Natural water	0.9 μg/L	82–102	<6.5	CE-electrochemical detection [31]
Water samples	40 μg/L	–	4.1	Photochemiluminometric [10]
Water samples	5 μg/L	–	1	FIA-fluorescence [8]
Soil	1 mg/L	–	–	TLC [32]
Peaches	43 μg/L	85–106	1.6	Synchronous fluorescence with derivatization [7]
Water samples	0.02 mg/L	93–96	1.16	Fluorometric [33]
Water samples	1.2×10^{-8} M	107	<5	FIA-amperometric [34]

ficient of $r = 0.994$ and R.S.D. = 0.78%. The detection limit (7.15 pM) was calculated as in Section 3.3. The mean recovery values of three separate determinations over 0.5 mL (50 mL reconstituted to 0.5 mL in the preconcentration step and transferred to a drive syringe of 5 mL) of water samples containing 50 pM spiked asulam was 98.14% with a R.S.D. = 1.90%.

The proposed method was compared with others described in literature; in Table 3 are depicted the analytical specifications of these methods [29]. As can be seen detection limit of the method described here is the best. Employing the compared techniques in natural waters, low recoveries are obtained [30]. It is known and well established that colloidal matter, suspended solids and dissolved organic material as humic substance may significantly affected the asulam partition and adsorption process from liquid to solid sorbent.

4. Conclusions

The kinetic information offered by the first instance (0.2 s) of the transient chemiluminescence signal from the system HRP–luminol–H_2O_2 when the pesticide asulam is present, allows us to perform a very sensitive analytical method for the determination of asulam at trace levels, based on the inhibition of the initial chemiluminescence of the base reaction. In addition to the high sensitivity, few interferences affect the method because data acquisition uses the initial steps of the chemiluminescent reaction and avoids side reaction from concomitant in the matrix having slower kinetic constants.

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