

Full Paper

Determination of selected nitrofurans using high performance liquid chromatography with post-column chemiluminescence detection

Sakchai Satienperakul^{1,*}, Chanyarat Tatana¹, Narin Taokaenchan², Wanida Sirikeaw^{3,4} and Saisunee Liawruangrath⁴

¹ Department of Chemistry, Faculty of Science, Maejo University, Chiang Mai 50290, Thailand

² Faculty of Agricultural Production, Maejo University, Chiang Mai 50290, Thailand

³ Samakkhi Witthayakhom School, Muang, Chiang Rai 5700, Thailand

⁴ Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai 52000, Thailand

* Corresponding author, e-mail: sakchais@mju.ac.th

Received: 7 April 2016 / Accepted: 4 November 2017 / Published: 16 November 2017

Abstract: Selected nitrofurans derivatives, specifically nitrofurazone, nitrofurantoin and furazolidone were determined by high performance liquid chromatography (HPLC) utilising a post-column chemiluminescence (CL) detection system. The CL reaction was based on the oxidation reaction between luminol and hydrogen peroxide in an alkaline medium and the enhancement of this reaction by nitrofurans. Optimum conditions were found: reagent stream of 1.5×10^{-3} M luminol reagent prepared in a mixed solution of 0.01 M sodium hydroxide and 1.0 M sodium chloride; oxidant/micellar solution of 7.5×10^{-3} M hydrogen peroxide in 0.1% Triton X-100; a mobile phase of acetonitrile : 10 mM acetate buffer, pH 6.0 (1:9 v/v); injection loop of 200 μ L; and analysis at room temperature. Under the optimal experimental conditions, linear calibration graphs of nitrofurazone, nitrofurantoin and furazolidone were obtained over concentration ranges of 0.1-5.0, 0.25-8.0 and 0.5-20.0 mg L⁻¹ respectively. Detection limits were found to be 0.1, 0.25 and 0.5 mg L⁻¹. The proposed HPLC-CL procedures were successfully applied to the determination of nitrofurans derivatives in pharmaceutical formulations, bee pollen and animal feeds.

Keywords: nitrofurans, high performance liquid chromatography, post-column, chemiluminescence detection, nitrofurazone, nitrofurantoin, furazolidone

INTRODUCTION

Nitrofurans are antimicrobial drugs which have been widely used as veterinary therapeutics in livestock, aquaculture and bee colonies in the prophylactic and therapeutic treatments of bacterial

and protozoan infections. Nitrofurans antibiotics were banned from use in the European Union in 1995 due to concerns about the carcinogenicity of their residues in edible tissues [1]. The most common nitrofurans used are nitrofurazone ([*(E)*-(5-nitrofuranyl)methylideneamino]urea; NFZ), nitrofurantoin (1-[*(E)*-(5-nitrofuranyl)methylideneamino]imidazolidine-2,4-dione; NFT) and furazolidone (3-[*(E)*-(5-nitrofuranyl)methylideneamino]-1,3-oxazolidin-2-one; FZD). Treatment of animals with these antibiotics may result in presence of nitrofurans residues in food products, leading to antibiotic resistance and allergic reactions in humans. The application and interpretation of the legislation on the control of exports from developing countries into Europe is somewhat complicated and has generated an increase in demand for screening assays of nitrofurans in exported food products.

The laboratory quality control usually deals with a large number of contaminated samples with a variety of residues to be detected in a short period of time. The use of rapid screening methods can improve the effectiveness of control in both official and industrial laboratories. An antibody-based method for nitrofurans detection, viz. enzyme-linked immunosorbent assay [2], provides a low cost, portable and high-throughput screening method capable of a sensitive determination of nitrofurans and their metabolites. Nevertheless, the contamination should be confirmed by a suitable instrumental method.

There are several analytical methods currently available for the determination of nitrofurans in pharmaceutical formulations. The US pharmacopeia recommends direct spectrophotometric measurements for NFZ and FZD while high performance liquid chromatography (HPLC) is suggested for the determination of NFT [3]. However, the British Pharmacopoeia recommends only spectrometric methods to quantify these nitrofurans [4].

Earlier methods for nitrofurans residue analysis in food products utilised HPLC with ultraviolet or photodiode array detection [5-8]. Due to a variety of complex matrices, however, this technique may not be specific enough to identify all analytes simultaneously. Liquid chromatography-mass spectrometry/mass spectrometry (LC-MS/MS) was then used as a confirmatory method for screening these compounds. The coupling of HPLC to tandem mass spectrometry (HPLC-MS/MS) has significantly advanced the capabilities of selective quantitation methods for nitrofurans in recent years [9-11]. However, this sophisticated technique requires comparatively expensive equipment and it is not readily amenable to creating cost-effective instrumentation.

Flow injection (FI) analysis has been widely applied to pharmaceutical analysis as well as other analytical fields. The popularity of FI analysis is rapidly growing as evidenced by the number of relevant scientific papers published every year [12]. With the aim of improving the sensitivity of FI determination, the use of chemiluminescence (CL) detection in conjunction with the FI technique has gained interest. The major advantage of CL reactions is that the sensitivity and selectivity in the determination of analytes can be improved since the absence of a light source diminishes noise and eliminates Rayleigh and Raman scattering, allowing the photon detectors to be operated at high gains to improve the signal-to-noise ratio. Additionally, CL has been applied in the FI determination of various inorganic and organic substances in food and pharmaceutical samples [13,14].

In early work on the determination of nitrofurans based on CL, only few reports have described the use of FI-CL for the determination of nitrofurans. Du et al. [15] reported the use of FI-CL for the determination of NFZ in pharmaceutical formulations and biological fluids based on the reaction of NFZ with H₂O₂ and N-bromosuccinimide in alkaline conditions. Li et al. [16] described a post-CL reaction of FZD with luminol-H₂O₂ CL reaction for the analysis of FZD in animal feeds.

Recently in our laboratory a simple acidic potassium permanganate CL was proposed for the determination of NFZ, NFT and FZD in animal feed [17]. These published findings claim that the proposed FI-CL method is more sensitive than the spectrophotometric ones and has advantages over other methods in that the consumption of samples, reagents and organic solvents, as well as waste generation is tremendously reduced. However, all of the three nitrofurans have not been analysed simultaneously.

Food samples often contain complex matrices and sometimes multiple analytes. The determination of trace levels of analytes has been limited by the inadequate selectivity and sensitivity provided by conventional liquid chromatographic detectors. Gámiz-Gracia et al. [18] recently reviewed advances in CL techniques in HPLC. A significant trend in the development is the search for more sensitive and selective methods. HPLC, in combination with sensitive post-column CL, has become quite a useful detection tool in recent years. Consequently, the application of CL in HPLC in the analysis of organic and inorganic species is widely investigated, particularly in clinical, pharmaceutical, environmental and food applications.

In the present paper we describe a further investigation of the CL detection system combined with classical reversed-phase HPLC for the analysis of nitrofurans (NFZ, NFT and FZD). The post-column CL detection procedure employed is based on the enhancing effect of the nitrofurans on luminal - hydrogen peroxide CL reaction in the presence of sodium chloride and Triton X-100 micelles, as similarly described by Serrano and Silva [19] and Perez-Ruiz et al. [20]. This determination avoids the use of derivatisation and provides a low limit of detection. A special feature of the CL detector is that it provides an alternative chromatographic procedure for screening and selective quantitation of nitrofurans residue in food and pharmaceutical samples after sample clean-up by solid-phase extraction (SPE) prior to analysis.

MATERIALS AND METHODS

Chemicals and Reagents

Purified water from a compact ultra-pure water system (18.2 M Ω , Millipore, France) was used for all solution preparations. All reagents were of analytical and chromatographic grades. The chemicals and reagents used were: NFZ, NFT, FZD, bentonite, carboxymethylcellulose, acacia, methylcellulose, polyethylene glycol, and starch (Sigma-Aldrich, Germany); luminol (Fluka, UK); *N,N*-dimethylformamide, Triton X-100, and acetonitrile (Fisher Scientific, UK); hydrogen peroxide and acetic acid (Merck, Germany); sodium hydroxide, sodium acetate, sodium chloride, fructose, glucose, lactose, sucrose, aluminum chloride, calcium chloride, magnesium chloride, potassium chloride, sodium sulphate, and sodium hydrogen phosphate (Ajax, Australia); sodium acetate (Carlo erba, Italy).

Standard solutions containing 100 mg mL⁻¹ of each antibiotic were prepared by dissolving the required amount of compound in 5.0 mL of *N,N*-dimethylformamide and diluting to 100 mL with ultra-pure water. Being unstable under UV radiation, the resulting dilute solutions of the nitrofurans were protected from light and stored refrigerated at 4°C. Stock working solutions were made by appropriate dilution of the standard solutions with deionised water or mobile phase (acetonitrile : 10 mM sodium acetate buffer pH 6.0, 1:9 v/v).

The reagent solution, consisting of luminol in alkaline medium, was prepared daily by diluting an appropriate amount of 0.01M luminol stock solution with a solution containing 0.01M sodium hydroxide and 1.0M sodium chloride. A mixture of 0.05% Triton X-100 and 0.05M H₂O₂,

used as the oxidant/micellar solution, was prepared by pipetting 25 mL of 1% Triton X-100 and 25 mL of 1.0M H₂O₂ into a 500-mL volumetric flask and diluting to the mark with deionised water. The mobile phase, containing acetonitrile : 10mM sodium acetate buffer at pH 6 (1:9 v/v), was prepared daily and functioned as a carrier solution for the post-column CL detection system.

All mobile phase and post-column reagent solutions were filtered through a 0.45- μ m nylon membrane filter (Trace Biotech, Australia) and degassed prior to use.

Samples and Sample Preparation

Samples

Commercial pharmaceutical formulations were purchased from local drug stores in Chiang Mai province, Thailand. These were: Furasian (Asian Pharmaceutical Ltd., Bangkok, each tablet containing 100 mg FZD); nitrofurantoin (A.N.H. Products Ltd., Bangkok, each tablet containing 100 mg NFT); and nitrofurazone ointment (Osoth Inter Laboratories Ltd., Bangkok; each 100 grams of the cream contains 200 mg NFZ).

Bee pollen from longan and four brands of animal feed were also purchased from local markets in Chiang Mai. All samples were kept refrigerated and protected from light as described previously by Hassan et al. [21] and Walash [22].

Pharmaceutical tablet preparation

Tablets (furasian or nitrofurantoin) were accurately weighed and ground into a fine powder. A sample amount equivalent to ca.20 mg of Furasian (or nitrofurantoin) was accurately weighed, transferred into a 100-mL volumetric flask and diluted to the mark with the mobile-phase solution. The solution was filtered through a nylon syringe filter (13 mm, 45 μ m) and then analysed by the HPLC-CL method (described later). Appropriate concentrations of sample solutions were obtained by dilution with the mobile phase prior to analysis.

NFZ ointment preparation

A quantity of NFZ ointment equivalent to 20.0 mg of NFZ was accurately weighed and dissolved in 10 mL of *N,N*-dimethylformamide by sonication for 35 min. The dissolved ointment base and nitro compound was then filtered and transferred to a 100-mL volumetric flask and the volume was made up with ultrapure water. Appropriate concentrations of sample solutions were obtained by dilution with the mobile phase prior to analysis.

Bee product preparation

SPE was used as an effective alternative to solvent extraction methods. It enables the analyte to be isolated and concentrated for testing. The use of two different SPE cartridges has been described earlier by Conneely et al. [23] and Barbosa, et al. [24]. Two grams of bee pollen sample was weighed accurately into a glass tube and 10 mL of the mobile-phase solution was added. The mixture was shaken manually followed by centrifugation at 3,000 rpm for 20 min. The upper layer of aqueous solution was passed through an SPE cartridge (Oasis® HLB 3cc, 60mg, Waters, USA).

Column preparation: An SPE column of hydrophilic-lipophilic-balance (HLB) copolymer was rinsed with 3 mL of methanol and the cartridge was then rinsed with 2 mL of deionised water at a flow rate of 1 mL min⁻¹.

Sample purification or clean-up process: A sample solution was applied at the top of the column and the solvent was drawn through the column bed using a syringe. The column was washed with 10 mL of 10 % methanol at a flow rate of 1 mL min⁻¹. The analyte fraction was then collected by eluting with 3 mL of methanol. The sample was dried under a gentle stream of pure nitrogen. Finally, the residue was dissolved in 1 mL of mobile-phase solution and the solution filtered through a 0.45- μ m membrane syringe filter, collected in a 10-mL glass vial and kept at 4°C before HPLC analysis. An appropriate concentration of the sample solution was obtained by dilution with the mobile-phase solution.

Animal feed preparation

Thoroughly minced feed (5.0 g) was added with 20 mL of ammonium acetate (79 mmol L⁻¹ solution, pH 4.6) and the pH was adjusted to 8 with diluted ammonia solution. The mixture was allowed to stand for 15 min., after which ethyl acetate (30 mL) was added. The mixture was stirred for 20 min. with a rotary shaker and centrifuged for 10 min. at 3000 rpm. The organic layer was collected and evaporated to dryness in a rotary vacuum evaporator at 35°C and 240 mbar. The resulting extract was reconstituted in 2 mL of acetone : methanol (8:2 v/v).

An SPE cartridge (Sep-Pak[®] NH₂ Vac, 6cc, 1g, Waters, USA) was conditioned with 5 mL of acetone : methanol (8:2 v/v). The reconstituted extract was put onto the cartridge and the nitrofurans were eluted with 5 mL of acetone : methanol (8:2 v/v). The eluate was evaporated to dryness and the residue was reconstituted with 5 mL of the mobile-phase solution. The resulting solution was filtered through a 0.45- μ m membrane syringe filter before being injected into the HPLC-CL system.

Apparatus

FI-CL analysis

The FI manifold used in this experiment is illustrated in Figure 1. The experimental set-up consisted of a two-channel peristaltic pump with a rate selector (Minipuls 3, Gilson, France), a sample injection valve (Type 50, Rheodyne, USA), and PTFE connection tubing (i.d. 0.5 mm, Alltech, USA). The CL signal was monitored with a custom-built flow-through luminometer that consisted of a flat spiral-glass flow cell (glass tubing, i.d. 1.5 mm, spiral coil diameter 25 mm) mounted flush against a photomultiplier tube (PMT) (Thorn-EMI model 9828SB, Electron Tubes, UK). The operational potential for the PMT was provided by a stable power supply (Thorn-EMI model PM20D, Electron Tubes, UK). The flow cell, PMT and voltage divider were encased in a light-tight housing. The detector output was recorded on a PC (Pentium IV) via a USB/RS-232 interface, with a multimeter (UNI-T, UT60F, Hong Kong) for determining the peak maximum. As the pump tubes change their properties with time, affecting the analytical signal, the flow rates in both channels were measured and adjusted regularly to maintain equal flow rates.

HPLC-CL analysis

A simplified scheme of the instrumental assembly used in HPLC with post-column CL detection is depicted in Figure 1. The chromatographic separations were performed using an HPLC system consisting of Agilent 1100 (Hewlett-Packard, Germany), Rheodyne 7725i with a 200- μ L injection loop, an isocratic pump G1310A, and an Eclipse XDB-C₁₈ column (4.6 \times 150 mm, 5 μ m). A flow-through luminometer as described earlier functioned as a post-column detector for the HPLC system.

A UV diode array detector (Hewlett-Packard, Germany) was employed as a comparison detector for the determination of nitrofurans in samples by measuring the absorbance at 375 nm [24]. A vortex (Touch Mixer model 232, Fisher Scientific, U.S.A.) and centrifuge (Centurion 1000 series, Labquip, UK) were used to prepare the sample solutions. All samples were filtered through 0.45- μm nylon syringe filters before HPLC analysis.

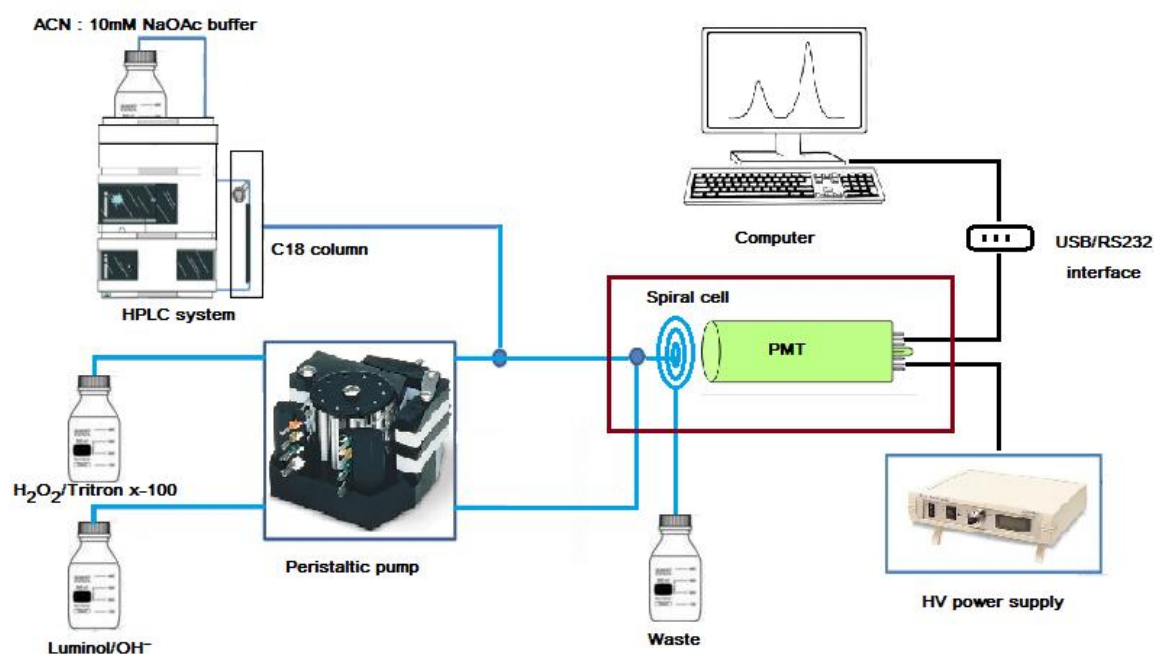


Figure 1. Schematic diagram of HPLC-CL system. (For experimental conditions, see Table 1.)

Procedures

Initially, the CL detector was tested using a flow injection manifold similar to that used in the previous work reported by Serrano and Silva [19]. The sensor performance, i.e. slope and linearity, was optimised by injecting nitrofurans standards and measuring their peak heights. The standards were injected into the water stream (flow rate 1.0 mL min^{-1}) and mixed with $0.05 \text{ M H}_2\text{O}_2$ in the presence of 0.05% Triton X-100 solution (flow rate 1.5 mL min^{-1}) as the oxidant/micellar stream. The reaction mixture was then merged at a T-piece with the reagent stream (flow rate 1.5 mL min^{-1}) consisting of 0.5 mM luminol in 0.01 M NaOH and 1.0 M NaCl . The flow of the combined reaction mixture at 4.5 mL min^{-1} was passed through a flat spiral-coil flow cell, where the elicited CL intensity was measured by the PMT operated at a voltage of 0.86 kV . The output of the PMT, which was proportional to the CL intensity, was monitored continuously.

The manifold used when the CL sensor was applied as detector in the HPLC is shown in Figure 1. In this case the eluent from the HPLC column (acetonitrile : 10 mM sodium acetate buffer $\text{pH } 6 = 1:9 \text{ v/v}$) was merged with the oxidant/micellar stream and was transported to the CL detector, which required 5-10 min. to obtain a stable baseline. In the HPLC-CL work, mixed standards containing NFZ, NFT and FZD were used.

Comparative chromatographic measurements were performed by an Agilent HPLC system consisting of an Agilent HPLC pump, a Rheodyne 7725 injector fitted with a $20\text{-}\mu\text{L}$ sample loop, and a diode array detector set at 375 nm . The analytical column was a Hypersil $5\text{-}\mu\text{m}$ ODS column ($125 \times 4.0 \text{ mm}$). The method was modified according to Barbosa et al. [24]. A binary gradient

mobile phase of 14mM ammonium acetate (pH 4.6) and acetonitrile was used at the flow rate of 0.6 mL min⁻¹. The official British Pharmacopoeia procedure [4] was performed via a Hitachi U-2001 spectrophotometer at the absorption wavelength of 375nm for NFZ and 367 nm for NFT and FZD.

RESULTS AND DISCUSSION

Optimisation of Experimental Variables for FI-CL

A series of experiments was conducted to establish the optimum experimental conditions that yielded high CL sensitivity. Optimisation of the FI system was conducted by the univariate methods. Injections in triplicate of each of the 1.0-mg L⁻¹ FZD, NFT and NFZ standards were evaluated for each set of parameters.

PMT voltage

The influence of PMT voltage was studied firstly to search for an optimal input voltage. The voltages ranging 750-1,000 V were tested, the maximum input voltage recommended by the manufacturer being 1,000 V. The experiments were performed with multiple injections of standard solutions of each nitrofurans (NFZ, NFT and FZD) at the concentration of 1.00 mg L⁻¹, made up in a carrier stream. The four-channel peristaltic pump was used to propel the carrier stream, reagent stream and oxidant/micellar stream. The total flow rate was set at 3.0 mL min⁻¹ (1.0 mL min⁻¹ each). The potential of the power supply was increased stepwise and the current representing CL intensity (in mV) was measured after an injection of NFZ, NFT or FZD at each potential step. As expected, both noise and analytical signals increased as the PMT voltage increased. The resulting plot of CL signal-to-noise ratio of all nitrofurans reached a maximum value at 860 V, which was selected for subsequent experiments.

Triton X-100 concentration

Due to the unique and advantageous properties of surfactants, the use of Triton X-100 in the oxidant/micellar stream should better facilitate analytical CL measurements. Previous papers by Serrano and Silva [19] and Perez-Ruiz et al. [20] reported the use of Triton X-100 surfactant in CL measurements based on its enhancing effect on luminol-H₂O₂ CL reaction. The influence of Triton X-100 on emission intensity in the luminol CL system was studied by dissolving 0.02M H₂O₂ in solutions of Triton X-100 at concentrations ranging between 0.001-0.1% by volume. It was observed that there were improvements in both signal intensity and signal-to-noise ratio in the determination of nitrofurans. The results agree well with previous reports [19, 20], with the most suitable Triton X-100 concentration for the FI-CL being 0.05%.

Hydrogen peroxide concentration

The dependence of the CL intensity on H₂O₂ was examined in the concentration range of 0.01-0.1M in order to find the optimum concentration for the FI-CL system. H₂O₂ was mixed with 0.05% Triton X-100 solution in deionised water and then used as the oxidant/micellar stream. The maximum CL intensity was observed when the H₂O₂ concentration was 0.05M. Above this concentration the CL intensity signal decreased and many gaseous bubbles appeared in the waste solution. Therefore 0.05M was chosen as the optimum H₂O₂ concentration.

Effect of different sensitisers on CL intensity

In some cases in the absence of a sensitiser, luminol systems can only produce weak CL emissions. Thus, various compounds such as quinine, fluorescein and rhodamine B were tested as potential sensitisers. It was found that all sensitisers showed little or no effect on the CL intensity. Hence it was not necessary to use these sensitisers to enhance the CL intensity of nitrofurans.

Luminol concentration

The influence of varying concentrations of luminol was studied by dissolving luminol in a mixture of 0.05M NaOH and 0.1M NaCl. Luminol concentrations between 1.0×10^{-4} - 1.5×10^{-3} M were examined. The maximum CL intensity was obtained when the concentration of luminol was 5.0×10^{-4} M, which was used in the further studies.

Sodium chloride and sodium hydroxide concentrations

The effect of NaCl concentration on the CL response was studied in the range 0.01-5.0M by dissolving the NaCl in 0.01M NaOH. The optimum signal was obtained when 1.0M NaCl was used. Because the CL signal could only be observed in alkaline medium [25], the strongest CL signal was obtained with 0.01M NaOH solution. This phenomenon can be explained by the fact that H_2O_2 decomposes in alkaline solution and oxidises nitrofurans, releasing energy which is accompanied by CL. However, by increasing the concentration of NaOH a high baseline was observed. This phenomenon could be due to the hydroxyl radical produced at a high basic concentration acting as a co-oxidant in the H_2O_2 -luminol system. The hydroxyl radical is a typical one-electron oxidant which can oxidise luminol to form a luminol radical intermediate, which is further oxidised by dissolved oxygen or hydrogen peroxide to yield an electronically excited 3-aminophthalate ion, which returns to the ground state producing CL. This leads to a shift of the signal baseline. Hence NaOH at 0.01M was chosen for the subsequent experiments because it gave the highest CL signal over the studied range and did not give a baseline fluctuation.

Flow rate

The time taken to transfer the excited product into the flow cell is another important factor in the maximum collection of the emitted light [26]. Effects of flow rate of the carrier stream, reagent stream and stream of oxidant/micellar solution were studied. The flow rate of the reagent solution was optimised in order to obtain satisfactory CL intensity. The peristaltic pump conveniently controlled the flow rates of both the reagent and oxidant/micellar streams, which were set equal to simplify the FI system and studied over the range of 0.3-3.0 mL min⁻¹. A flow rate of 1.5 mL min⁻¹ was determined to produce optimum CL signal. Apparently at a lower flow rate, the reaction slowly occurred, causing an incomplete reaction and a low signal. At flow rates above 1.5 mL min⁻¹ the CL intensity diminished gradually due to a shorter time interval between mixing and observing CL and poorer CL reactivity occurring in the reaction zone of the flow cell.

The deionised water carrier flow rate was found to have little effect on the response of the CL sensor within the range of 0.5-1.5 mL min⁻¹. The CL intensity slightly increased with the flow rate up to 1.0 mL min⁻¹ and remained almost constant above this value. Therefore in this preliminary investigation the carrier flow rate of 1.0 mL min⁻¹ was chosen.

Injection volume

An increase in sample volume normally leads to an increase in the emitted CL signal. The effect of the sample/standard volume on the CL intensity was investigated by injecting each standard solution (1.0 mg L⁻¹ NFZ, NFT and FZD) of varying volumes in the range of 50-500 µL. With an injection value of 50 µL, the lowest CL intensity was obtained. With volumes greater than 200 µL, peak broadening was observed. Therefore the injection volume of 200 µL was selected since it gave a good sensitivity, reproducibility and reasonable sample throughput.

Sensitivity

Under the optimum conditions, the sensitivity of the developed FI-CL for NFZ was the highest at 15.70 mV mg⁻¹ L (R² = 0.9926); that for NFT and FZT was 10.79 mV mg⁻¹ L (R² = 0.9998) and 5.34 mV mg⁻¹ L (R² = 0.9995) respectively.

Application of CL Detection in HPLC

A further optimisation study of the reagent conditions for HPLC-CL was investigated to achieve a sensitive post-column detection system after chromatographic separation of the nitrofurans residues based on the aforementioned FI-CL system. The choice of chromatographic conditions that ensure resolved peaks for the analytes was based on previous work by McCracken and Kennedy [27]. In this work similar eluent conditions were used to achieve a good analytical separation using a C₁₈ column for the mixture of NFZ, NFT and FZD. The ammonium acetate or sodium acetate buffer in the mobile phase had only a slight influence on the CL response; however, the baseline was more stable and the peaks were well-separated and reasonably-shaped when acetonitrile : 10 mM sodium acetate (1:9) was employed [17, 24].

In addition to the composition of the mobile phase, the effect of its flow rate on the chromatographic resolution was examined in order to obtain a reasonable analysis time (<30 min.). The CL detection system parameters were also re-evaluated in terms of their performance in achieving maximum sensitivity along with good analytical separations. All optimum values were chosen subjectively by compromising amongst the peak height, stability of the base line, low or no-positive blank signals and short analysis time. The working ranges within which these parameters were optimised and the corresponding optimal values are presented in Table 1.

Under the chromatographic separation and CL sensor conditions acquired as mentioned above, a typical chromatogram (Figure 2) resulting from the injection of a synthetic mixture of NFZ (4.0 mg L⁻¹), NFT (6.0 mg L⁻¹) and FZD (8.0 mg L⁻¹) was obtained. The three derivatives were completely resolved from each other within the run-time of 20 min.

Analytical Calibration and Merits

Calibration graphs for the three nitrofurans were constructed using the least-square regression of different amounts of each nitrofurans standard (NFZ, NFT and FZD) in the mobile phase versus peak heights under selected experimental conditions (Table 1). At least 6 samples covering the whole range were used. Each point of the calibration graph corresponds to the mean value from three independent peak measurements. The results obtained are summarised in Table 2, showing the least-square parameters of the working curves and retention time. The limit of detection (3σ) and limit of quantitation (10σ) were also determined using the lowest concentration of each nitrofurans and measuring the signal-to-noise ratio from the resulting chromatograms.

Unsurprisingly, the sensitivities (slopes) were lower than those obtained previously when assessing the performance of the CL detector by FI. This is obviously due to a greater sample dispersion in the HPLC, which leads to higher detectable concentrations of the residual compounds. Furthermore, comparison of the proposed HPLC-CL method with selected methods reported earlier for determining nitrofurans (Table 3) indicated that the proposed method is sensitive and inexpensive while providing a selective screening procedure for nitrofurans determination.

Table 1. Optimised HPLC-CL system parameters compared with FI-CL values

Parameter	Range studied	HPLC-CL value	FI-CL value
Mobile phase			
Composition	-	CH ₃ CN: NaOAc = 1:9	Water
Flow rate (mL min ⁻¹)	0.5-1.5	1.2	1.0
Oxidant/micellar stream solution			
Hydrogen peroxide concentration (M)	5.0×10 ⁻³ -0.10	7.5×10 ⁻³	0.05
Triton X-100 concentration (%)	0.001-0.1	0.1	0.05
Flow rate (mL min ⁻¹)	1.0-2.0	1.5	1.5
Reagent stream solution			
Luminol concentration (M)	5.0×10 ⁻⁴ -2.0×10 ⁻³	1.5 ×10 ⁻³	5.0 ×10 ⁻⁴
Sodium hydroxide concentration (M)	5.0×10 ⁻³ -0.01	0.01	0.01
Sodium chloride concentration (M)	0.01-5.0	1.0	1.0
Flow rate (mL min ⁻¹)	0.3-3.0	1.5	1.5
PMT voltage (V)	-	860	860
Injection volume (μL)	-	200	200
Analytical column	-	C ₁₈ , 5μm, 4.6 mm×150mm	-

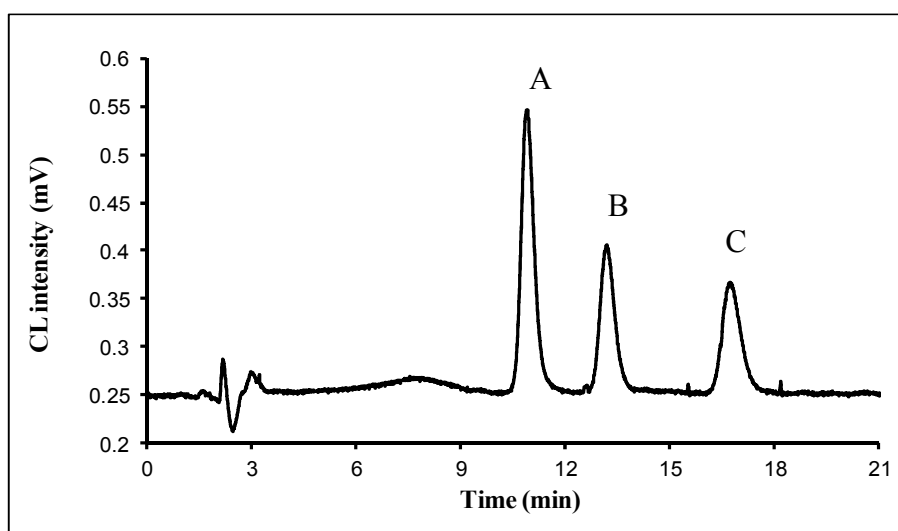


Figure 2. Chromatogram of a synthetic mixture (200 μL) of (A) NFZ (4.0 mg L⁻¹), (B) NFT (6.0 mg L⁻¹) and (C) FZD (8.0 mg L⁻¹). For experimental conditions, refer to Table 1.

Table 2. Analytical merits of NFZ, NFT and FZD by HPLC-CL systems

Analytical merit	NFZ	NFT	FZD
Working range (mg L ⁻¹)	0.1-5.0	0.25-8.0	0.5-20.0
Linear regression equation	$y=9.347x + 0.502$ (R ² = 0.995)	$y=6.860x + 0.789$ (R ² = 0.998)	$y=4.058x + 1.763$ (R ² = 0.997)
Retention time (min.)	t _{R1} = 10.31	t _{R2} = 13.57	t _{R3} = 17.65
Limit of detection (mg L ⁻¹)	0.1	0.25	0.5
Limit of quantitation (mg L ⁻¹)	0.9	0.6	1.0

Table 3. Comparison of analytical figures of merit of proposed HPLC-CL method with earlier reported methods

Compound	Technique	Linear range (mg L ⁻¹)	LOD* (mg L ⁻¹)	Sample	Reference
NFZ	FI-CL (N-bromosuccinimide - H ₂ O ₂)	0.1-100	0.02	Pharmaceutical preparations Blood plasma Urine	[15]
FZD	FI-CL (Luminol - H ₂ O ₂)	0.1-10	0.0196	Animal feeds	[16]
NFZ NFT FZD	FI-CL (Acidic KMnO ₄)	0.5 - 8	0.25 0.25 0.25	Animal feeds	[17]
FZD	HPLC-UV (365 nm)	5-125	5	Animal feeds	[27]
NFZ FZD	HPLC-DAD	0.1-2.5	2.5 × 10 ⁻³ 5.0 × 10 ⁻³	Avian egg	[7]
NFZ NFT FZD FTD	LC-MS/MS	20 × 10 ⁻³ - 50 × 10 ⁻³	7-21 × 10 ⁻³	Animal feeds	[24]
NFZ NFT FZD	HPLC-CL	0.1-5.0 0.25-8.0 0.5-20.0	0.1 0.25 0.5	Pharmaceutical preparations Bee pollen Animal feeds	This work

* Limit of detection

Effect of Potential Interferences

Possible interferences of common excipients (acacia, bentonite, carboxymethyl cellulose, fructose, glucose, lactose, methyl cellulose, polyethylene glycol, starch, sucrose), some cations (Al³⁺, Ca²⁺, Mg²⁺, Na⁺, K⁺) and anions (Cl⁻, SO₄²⁻, HPO₄²⁻), which might concurrently be present in pharmaceutical preparations and animal feed samples, were investigated via the FI-CL procedure. A foreign substance was considered not interfering if it caused a relative error of less than 5% for 2 mg L⁻¹ of nitrofurans. The maximum tolerable concentrations of each excipient and common ion for 2 mg L⁻¹ of nitrofurans were over 1000-fold for sucrose, lactose, acacia, propylene glycol or starch; 100-fold for glucose, fructose, sodium carboxymethyl cellulose, Cl⁻, SO₄²⁻, HPO₄²⁻, K⁺, Na⁺, Ca²⁺ or Mg²⁺; and 10-fold for Al³⁺, methyl cellulose or bentonite.

After SPE, the HPLC chromatograms of real samples have clear baselines with no interferences to the analyte peaks. Hence it can be concluded that all excipients show no serious effect on the determination of nitrofurans even though they are present at 10-1,000 times the weight of nitrofurans.

Analysis of Samples

In order to evaluate the accuracy of the method, two antiseptic tablet samples, an antiseptic ointment, bee pollen, and four samples of animal feed were analysed by the proposed HPLC-CL procedure under optimum experimental conditions.

The results of the determination of NFZ, NFT and FZD in the pharmaceutical preparations using HPLC-CL are given in Table 4. The good agreement of the HPCL-CL results with the product labels and values obtained from the BP standard method [4] is clearly seen.

Results are also given for bee pollen and animal feeds (Table 4), compared with those by HPLC with UV-DAD detector. There is no significant difference between the mean values obtained by both methods at 95% confidence (t-test). The analytical accuracy of the proposed method was evaluated by determining the recoveries of nitrofurans after spiking five known amounts of each nitrofuran in bee pollen and animal feed samples. The recoveries of nitrofurans by this method showed satisfactory results ranging between 93.0-105.6%.

Table 4. Comparative determinations of nitrofurans in real samples by proposed HPLC-CL method and standard BP method or HPLC-DAD method

Sample	Concentration									<i>t</i> value *
	NFZ			NFT			FZD			
	Declared	Reference method	HPLC-CL method	Declared	Reference method	HPLC-CL method	Declared	Reference method	HPLC-CL method	
Nitrofurazone ointment (mg kg ⁻¹)	-	170.37 ± 6.17 ^a	166.04 ± 9.87	-	-	-	-	-	-	1.87
Nitrofurantoin Tablet (mg/Tablet)	-	-	-	100	99.47 ± 1.10 ^a	104.33 ± 4.66	-	-	-	1.76
Furazolidone Tablet (mg/Tablet)	-	-	-	-	-	-	100	96.38 ± 0.39 ^a	98.03 ± 3.65	1.42
Bee pollen (mg kg ⁻¹)	-	ND ^c	ND	-	0.175 ± 0.03 ^b	0.198 ± 0.02	-	ND	ND	1.10
Feed A (mg kg ⁻¹)	-	ND	ND	-	ND	ND	-	3.71 ± 0.01 ^b	3.50 ± 0.03	1.84
Feed B (mg kg ⁻¹)	-	ND	ND	-	ND	ND	-	2.87 ± 0.01 ^b	3.01 ± 0.03	1.24
Feed C (mg kg ⁻¹)	-	ND	ND	-	ND	ND	-	0.47 ± 0.01 ^b	0.32 ± 0.03	1.21
Feed D (mg kg ⁻¹)	-	ND	ND	-	ND	ND	-	0.31 ± 0.01 ^b	0.47 ± 0.03	1.33

Note: All measurements were conducted in triplicate (± SD)

* *t*-critical = 2.776 at 95% confidence

^a The official British Pharmacopoeia method [4]

^b HPLC-DAD method [24]

^c Not detectable

CONCLUSIONS

The CL detector has been successfully applied in conjunction with HPLC for the determination of nitrofurans. Analytical results have indicated that the CL post-column system is suitable as a sensitive detector for HPLC and that potential interferences from metal ions in real samples can be sufficiently eliminated by SPE cleanup prior to performing the analysis. Thus, the proposed HPLC-CL method is valid and can be an alternative and relatively selective screening procedure for identifying and eliminating the contamination sources in order to ensure the chemical safety of foods available to consumers.

ACKNOWLEDGEMENTS

C. T. and N. T. gratefully acknowledge Thailand Research Fund-Master Research Grants (TRF-Mag) for financial assistance. The authors thank members of the Central Laboratory (Thailand) Co. Ltd., Chiang Mai for their valuable consulting.

REFERENCES

1. M. Vass, K. Hruska and M. Franek, "Nitrofurantoin antibiotics: A review on the application, prohibition and residual analysis", *Vet. Med.*, **2008**, *53*, 469-500.
2. M. O'Keefe, A. Conneely, K. M. Cooper, D. G. Kennedy, L. Kovacsics, A. Fodor, P. P. J. Mulder, J. A. van Rhijn and G. Trigueros, "Nitrofurantoin antibiotic residues in pork, the food BRAND retail survey", *Anal. Chim. Acta*, **2004**, *520*, 125-131.
3. US Pharmacopoeia, "The United States Pharmacopoeia 30 and the National Formulary 25 Second Supplement", USP Convention, Rockville, **2007**.
4. British Pharmacopoeia Commission, "British Pharmacopoeia, Vol. 2", The Stationery Office, London, **2007**.
5. L. Kumar, J. R. Toothill and K. B. Ho, "Determination of nitrofurantoin residues in poultry muscle tissues and eggs by liquid chromatography", *J. AOAC Int.*, **1994**, *77*, 591-595.
6. K. Yoshida and F. Kondo, "Liquid chromatographic determination of furazolidone in swine serum and avian egg", *J. AOAC Int.*, **1995**, *78*, 1126-1129.
7. R. Draisci, L. Giannetti, L. Lucentini, L. Palleschi, G. Brambilla, L. Serpe and P. Gallo, "Determination of nitrofurantoin residues in avian eggs by liquid chromatography-UV photodiode array detection and confirmation by liquid chromatography-ionspray mass spectrometry", *J. Chromatogr. A*, **1997**, *777*, 201-211.
8. N. M. Angelini, O. D. Rampini and H. Mugica, "Liquid chromatographic determination of nitrofurantoin residues in bovine muscle tissues", *J. AOAC Int.*, **1997**, *80*, 481-485.
9. G. Balizs and A. Hewitt, "Determination of veterinary drug residues by liquid chromatography and tandem mass spectrometry", *Anal. Chim. Acta*, **2003**, *492*, 105-131.
10. E. Verdon, P. Couedor and P. Sanders, "Multi-residue monitoring for the simultaneous determination of five nitrofurans (furazolidone, furaltadone, nitrofurantoin, nitrofurantoin, nifursol) in poultry muscle tissue through the detection of their five major metabolites (AOZ, AMOZ, SEM, AHD, DNSAH) by liquid chromatography coupled to electrospray tandem mass spectrometry—In-house validation in line with Commission Decision 657/2002/EC", *Anal. Chim. Acta*, **2007**, *586*, 336-347.
11. M. I. Lopez, M. F. Feldlaufer, A. D. Williams and P-S. Chu, "Determination and confirmation of nitrofurantoin residues in honey using LC-MS/MS", *J. Agri. Food Chem.*, **2007**, *55*, 1103-1108.

12. J. Ruzicka and E. H. Hansen, "Flow Injection Analysis", 2nd Edn., John Willey and Sons, New York, **2003**.
13. M. J. Navas and A. M. Jiménez, "Review of chemiluminescent methods in food analysis", *Food Chem.*, **1996**, *55*, 7-15.
14. Y. F. Mestre, L. L. Zamora and J. M. Calatayud, "Flow-chemiluminescence: A growing modality of pharmaceutical analysis", *Luminescence*, **2001**, *16*, 213-235.
15. J. Du, L. Hao, Y. Li and J. Lu, "Flow injection chemiluminescence determination of nitrofurazone in pharmaceutical preparations and biological fluids based on oxidation by singlet oxygen generated in N-bromosuccinimide-hydrogen peroxide reaction", *Anal. Chim. Acta*, **2007**, *582*, 98-102.
16. X. Li, J. Hu and H. Han, "Flow-injection post-chemiluminescence determination of furazolidone in animal feeds", *Am. J. Biomed. Sci.*, **2009**, *1*, 260-266.
17. P. Thongsrisomboon, B. Liawruangrath, S. Liawruangrath and S. Satienerakul, "Determination of nitrofurans residues in animal feeds by flow injection chemiluminescence procedure", *Food Chem.*, **2010**, *123*, 834-839.
18. L. Gámiz-Gracia, A. M. García-Campaña, J. F. Huertas-Pérezb and F. J. Lara, "Chemiluminescence detection in liquid chromatography: Applications to clinical, pharmaceutical, environmental and food analysis--A review", *Anal. Chim. Acta*, **2009**, *640*, 7-28.
19. J. M. Serrano and M. Silva, "Rapid and sensitive determination of aminoglycoside antibiotics in water samples using a strong cation-exchange chromatography non-derivatisation method with chemiluminescence detection", *J. Chromatogr. A*, **2006**, *1117*, 176-183.
20. T. Perez-Ruiz, C. Martinez-Lozano, V. Tomas and J. Martin, "High-performance liquid chromatographic separation and quantification of citric, lactic, malic, oxalic and tartaric acids using a post-column photochemical reaction and chemiluminescence detection", *J. Chromatogr. A*, **2004**, *1026*, 57-64.
21. S. M. Hassan, F. A. Ibrahim, M. S. El-Din and M. M. Hefnawy, "A stability-indicating high-performance liquid chromatographic assay for the determination of some pharmaceutically important nitrocompounds", *Chromatographia*, **1990**, *30*, 176-180.
22. M. I. Walsh, A. M. El-Brashy and M. A. Sultan, "Colorimetric determination of some aromatic nitrocompounds of pharmaceutical interest", *Anal. Lett.*, **1993**, *26*, 499-512.
23. A. Conneely, A. Nugent, M. O'Keeffe, P. P. J. Mulder, J. A. van Rhijn, L. Kovacsics, A. Fodor, R. J. McCracken and D. G. Kennedy, "Isolation of bound residues of nitrofurans from tissue by solid-phase extraction with determination by liquid chromatography with UV and tandem mass spectrometric detection", *Anal. Chim. Acta*, **2003**, *483*, 91-98.
24. J. Barbosa, S. Moura, R. Barbosa, F. Ramos and M. I. N. Da Silveira, "Determination of nitrofurans in animal feeds by liquid chromatography-UV photodiode array detection and liquid chromatography - ionspray tandem mass spectrometry", *Anal. Chim. Acta*, **2007**, *586*, 359-365.
25. L. L. Klopff and T. A. Nieman, "Effect of iron(II), cobalt(II), copper(II), and manganese(II) on the chemiluminescence of luminol in the absence of hydrogen peroxide", *Anal. Chem.*, **1983**, *55*, 1080-1083.
26. A. M. Garcia-Campana and W. R. G. Baeyens, "Chemiluminescence in Analytical Chemistry", Marcel Dekker, New York, **2001**.

27. R. J. McCracken and D. G. Kennedy, "Determination of furazolidone in animal feeds using liquid chromatography with UV and thermospray mass spectrometric detection", *J. Chromatogr. A*, **1997**, *771*, 349-354.
28. R. J. McCracken, W. J. Blanchflower, C. Rowan, M. A. McCoy and D. G. Kennedy, "Determination of furazolidone in porcine tissue using thermospray liquid chromatography-mass spectrometry and a study of the pharmacokinetics and stability of its residues", *Analyst*, **1995**, *120*, 2347-2351.