

# 科技部補助專題研究計畫成果報告 期末報告

## 磁固相萃取方法的發展(第3年)

計畫類別：個別型計畫  
計畫編號：NSC 100-2113-M-029-002-MY3  
執行期間：102年08月01日至103年07月31日  
執行單位：東海大學化學系

計畫主持人：黃承文

計畫參與人員：學士級-專任助理人員：洪岑佩  
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中華民國 103年08月14日

中文摘要：近年來開發僅使用極少量有機溶劑或完全不需溶劑的微萃取方法是一項熱門的研究主題。在各種微萃取技術發展當中，使用表面修飾具特定選擇性基團或包覆特定金屬氧化物的磁性微米/奈米顆粒當作萃取靜相（即所謂的磁固相萃取）受到廣泛重視，特別是在生化、生物及環境分析領域。與其他萃取方法比較，磁固相萃取的最大優點是操作步驟非常簡單。將磁性顆粒與樣品溶液混合後，只需藉助一塊磁鐵即可分離表面吸附有目標分析物的磁性顆粒與溶液基質，不需複雜昂貴的分離裝置，過濾、離心等處理手續皆可省略。其他優點包括：微米或奈米顆粒表面積大，萃取效率高；原料價格便宜，顆粒製備程序簡單；可依需求彈性修飾顆粒表面基團。本計畫以含磷酸基藥物分子（單磷酸基的 glyphosate；雙磷酸基的 alendronate 與 ibandronate）為測試對象，發展磁固相萃取濃縮方法。計畫分三年進行，陸續完成：（1）以毛細管電泳/電激發化學發光法配合 Fe<sub>3</sub>O<sub>4</sub>@Al<sub>2</sub>O<sub>3</sub> 磁奈米粒子固相萃取分析藥物及尿液樣品中的 ibandronate；（2）以毛細管電泳/螢光偵測法配合 Fe<sub>3</sub>O<sub>4</sub>@Al<sub>2</sub>O<sub>3</sub> 磁奈米粒子固相萃取分析尿液與血漿樣品中的 alendronate；（3）以流動注入法配合電激發化學發光快速篩檢水中 glyphosate 除草劑。三項研究成果皆已發表於分析化學領域國際期刊。

中文關鍵詞：磁固相萃取；Fe<sub>3</sub>O<sub>4</sub>@Al<sub>2</sub>O<sub>3</sub> 奈米顆粒；毛細管電泳；ibandronate；alendronate；glyphosate

英文摘要：

英文關鍵詞：

# 科技部補助專題研究計畫成果報告

(期中進度報告/期末報告)

## 磁固相萃取方法的發展

計畫類別：個別型計畫 整合型計畫

計畫編號：NSC 100-2113-M-029-002-MY3

執行期間：100年8月1日至103年7月31日

執行機構及系所：東海大學化學系

計畫主持人：黃承文

共同主持人：

計畫參與人員：莊皓雲、洪岑佩、樓元琪

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出席國際學術會議心得報告

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中華民國 103 年 8 月 20 日

## 中文摘要

近年來開發僅使用極少量有機溶劑或完全不需溶劑的微萃取方法是一項熱門的研究主題。在各種微萃取技術發展當中，使用表面修飾具特定選擇性基團或包覆特定金屬氧化物的磁性微米/奈米顆粒當作萃取靜相（即所謂的磁固相萃取）受到廣泛重視，特別是在生化、生物及環境分析領域。與其他萃取方法比較，磁固相萃取的最大優點是操作步驟非常簡單。將磁性顆粒與樣品溶液混合後，只需藉助一塊磁鐵即可分離表面吸附有目標分析物的磁性顆粒與溶液基質，不需複雜昂貴的分離裝置，過濾、離心等處理手續皆可省略。其他優點包括：微米或奈米顆粒表面積大，萃取效率高；原料價格便宜，顆粒製備程序簡單；可依需求彈性修飾顆粒表面基團。本計畫以含磷酸基藥物分子（單磷酸基的 glyphosate；雙磷酸基的 alendronate 與 ibandronate）為測試對象，發展磁固相萃取濃縮方法。計畫分三年進行，陸續完成：（1）以毛細管電泳/電激發化學發光法配合  $\text{Fe}_3\text{O}_4@\text{Al}_2\text{O}_3$  磁奈米粒子固相萃取分析藥物及尿液樣品中的 ibandronate；（2）以毛細管電泳/螢光偵測法配合  $\text{Fe}_3\text{O}_4@\text{Al}_2\text{O}_3$  磁奈米粒子固相萃取分析尿液與血漿樣品中的 alendronate；（3）以流動注入法配合電激發化學發光快速篩檢水中 glyphosate 除草劑。三項研究成果皆已發表於分析化學領域國際期刊。

**關鍵詞：**磁固相萃取； $\text{Fe}_3\text{O}_4@\text{Al}_2\text{O}_3$  奈米顆粒；毛細管電泳；ibandronate；alendronate；glyphosate

## 英文摘要

In recent years, the development of microextraction techniques using very little or no solvent at all has attracted much attention. Several new microextraction methods, e.g. liquid-phase microextraction, microscale solid-phase extraction and solid-phase microextraction, have been successfully developed. One of those new techniques, the magnetic solid-phase extraction (MSPE) in which the extraction solid phase consists of magnetic micro- or nanoparticles immobilized with selective functional groups or metal oxides film on the surface, has initiated significant interest, particularly in the fields of biochemistry, biology, and environmental analysis. In comparison with other extraction methods, the major advantage of MSPE is its simple operation. After mixing the magnetic nanoparticles with the sample solution, separation of the analyte-adsorbed solid particles from the mother liquid can be easily performed using a magnet. Neither filtration nor centrifuge is required. Other advantages of MSPE include: high extraction efficiency due to large surface area of nanoparticles; low cost and simple process of magnetic particle preparation; versatile selection of immobilized functional groups.

In this research, we mainly develop new MSPE methods for some phosphate-bearing compounds (e.g. monophosphate pesticides: glyphosate; biphosphonate pharmaceuticals: alendronate and ibandronate). During the 3-year period, we have successfully completed the following works: (1) developing a CE-electrochemiluminescence method coupled with MSPE for the analysis of ibandronate in drug formulations and human urine; (2) developing an analytical method for alendronate in human urine and plasma by MSPE and CE-fluorescence detection; (3) developing a simple and rapid screening method for glyphosate in water using flow-injection with

electrochemiluminescence detection. The results of the research have been published in international journals.

**Keywords:** magnetic solid-phase extraction; Fe<sub>2</sub>O<sub>3</sub>@Al<sub>2</sub>O<sub>3</sub> nanoparticles; capillary electrophoresis; ibandronate ; alendronate ; glyphosate

### 報告內容

1. 第一年研究成果：以毛細管電泳/電激發化學發光偵測配合磁固相萃取分析藥物與尿液中的雙磷酸鹽藥物 ibandronate，發表於 *Electrophoresis* 2011, 32, 2155-2160.
2. 第二年研究成果：以毛細管電泳/螢光偵測配合磁固相萃取分析血漿與尿液中的雙磷酸鹽藥物 alendronate，發表於 *J. Sep. Sci.* 2012, 35, 681-687.
3. 第三年研究成果：以流動注入/電激發化學發光偵測配合磁固相萃取快速篩檢水中微量除草劑 glyphosate，發表於 *Anal. Methods* 2013, 5, 6186-6191..

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Received February 28, 2011

Revised April 2, 2011

Accepted April 15, 2011

## Research Article

# Capillary electrophoresis– electrochemiluminescence detection method for the analysis of ibandronate in drug formulations and human urine

A simple, rapid and sensitive CE method coupled with electrochemiluminescence (ECL) detection for direct analysis of ibandronate (IBAN) has been developed. Using a buffer solution of 20 mM sodium phosphate (pH 9.0) and a voltage of 13.5 kV, separation of IBAN in a 30-cm length capillary was achieved in 3 min. ECL detection was performed with an indium tin oxide working electrode bias at 1.6 V (versus a Pt wire reference) in a 200-mM sodium phosphate buffer (pH 8.0) containing 3.5 mM Ru(bpy)<sub>3</sub><sup>2+</sup> (where bpy = 2,2'-bipyridyl). Derivatization of IBAN prior to CE-ECL analysis was not needed. Linear correlation ( $r = 0.9992$ ,  $n = 7$ ) between ECL intensity and analyte concentration was obtained in the range of 0.25–50  $\mu$ M IBAN. The LOD of IBAN in water was 0.08  $\mu$ M. The developed method was applied to the analysis of IBAN in a drug formulation and human urine sample. SPE using magnetic Fe<sub>3</sub>O<sub>4</sub>@Al<sub>2</sub>O<sub>3</sub> nanoparticles as the extraction phase was employed to pretreat the urine sample before CE-ECL analysis. The linear range was 0.2–12.0  $\mu$ M IBAN in human urine ( $r = 0.9974$ ,  $n = 6$ ). The LOD of IBAN in urine was 0.06  $\mu$ M. Total analysis time including sample preparation was <1 h.

### Keywords:

CE / Electrochemiluminescence detection / Ibandronate / Magnetic SPE / Urine analysis  
DOI 10.1002/eips.201100202

## 1 Introduction

Ibandronate sodium ([1-hydroxy-3-(methylpentylamino)propylidene]bisphosphonic acid monosodium; IBAN) (Fig. 1), a relatively new nitrogen-containing bisphosphonate drug, is a third-generation bisphosphonate for the treatment of bone disease such as malignant hypercalcemia and postmenopausal osteoporosis [1, 2]. Its once-monthly oral and quarterly intravenous (i.v.) dosage regimens have the potential to improve treatment adherence and persistence, and hence clinical outcomes, compared with other frequently administered oral bisphosphonates. Like other bisphosphonates, IBAN inhibits osteoclast-mediated bone resorption. Due to the stability of the P-C-P backbone and their polar nature, IBAN does not undergo metabolic biotransformation following oral administration and i.v. injection. It has been reported

that, in a mass balance study, nearly the entire oral IBAN dose was excreted unchanged in the feces, and the majority of i.v. IBAN was excreted unchanged in the urine within 24 h [3]. For comprehensive studies of the pharmacology and pharmacodynamic effects of IBAN in biological matrices, a simple, sensitive and accurate assay is generally required.

Analysis of IBAN represents a challenge because of its high polarity and low volatility. Furthermore, IBAN possesses no chromophore or fluorophore, and its tertiary amine characteristic renders derivatization difficult. Analytical methods for IBAN reported to date are therefore limited. A highly sensitive GC-MS assay for IBAN in serum/plasma and urine was first described by Endeke et al. [4]. The quantification limits could reach low ng/mL level. However, sample preparation was lengthy and tedious. Besides, in order to lower IBAN's polarity and enhance its volatility, derivatization into methyl ester prior to GC separation was required. A simple, non-derivatization detection method, evaporative light-scattering detection (ELSD), coupled with ion-pair HPLC analysis of IBAN was reported by Jiang and Xie [5]. The main drawback of this method is its relatively high LOD of IBAN (176  $\mu$ g/mL). A post-column photochemical derivatization followed by sensitive fluorometric detection for HPLC determination of several bisphosphonate drugs including IBAN in human urine has been reported by Pérez-Ruiz et al. [6]. Detection was based on their oxidation to orthophosphate by the on-line peroxydisulfate-assisted

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**Abbreviations:** CV, cyclic voltammetry; ECL, electrochemiluminescence; Fe<sub>3</sub>O<sub>4</sub>@Al<sub>2</sub>O<sub>3</sub> NPs, alumina-coated iron oxide nanoparticles; IBAN, ibandronate; ITO, indium tin oxide; Ru(bpy)<sub>3</sub><sup>2+</sup>, tris(2,2'-bipyridyl)ruthenium(II)

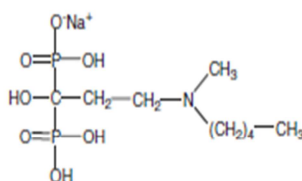


Figure 1. Molecular structure of ibandronate sodium.

photolysis followed by post-column reaction with molybdate to yield phosphomolybdate. This subsequently reacted with thiamine to generate thiochrome and, finally, the fluorescence of thiochrome was measured. This detection strategy is in the nature of indirect detection mode. The LOD of IBAN was 72 nM ( $\sim 23$  ng/mL). A high-throughput HPLC-MS/MS method for determining IBAN in human plasma has been developed by Tarcomnicu et al. [7]. Liquid-liquid extraction and methylation derivatization were used in sample preparation. The LOQ was 0.2 ng/mL. Recently, a simple ion chromatography (IC) method for the simultaneous determination of IBAN drug and its impurities has been reported by Narendra Kumar et al. [8]. The LOD of IBAN was 38  $\mu$ g/mL. ELISA, the most sensitive method for IBAN to date with a LOD at pg/mL level, also has been reported [4], but its reagents were not readily available.

CE has been a popular technique for drug analysis because of its high efficiency and versatility, very low sample and reagent consumption, low cost and minimization of environmental pollution. However, to our knowledge, there has been no CE method reported for the analysis of IBAN yet, which might be attributed to the lack of sensitive detection method for IBAN. There was a report on the use of CE-indirect UV detection for analyzing IBAN-related impurities, i.e. phosphite and phosphate, but IBAN itself was not determined [9]. In recent years, tris(2,2'-bipyridyl)ruthenium(II) ( $\text{Ru}(\text{bpy})_3^{2+}$ )-based electrochemiluminescence (ECL) has become an important and powerful detection method for CE due to its high sensitivity and simple instrumental setup [10]. It is known that amines, particularly tertiary amines, can be directly determined by utilizing their ECL reaction with electrogenerated  $\text{Ru}(\text{bpy})_3^{2+}$  without the need of derivatization [11, 12]. Since IBAN structurally belongs to a tertiary amine, we expect that it should be feasible to analyze IBAN with the CE-ECL method.

In this paper, we describe a simple, rapid and sensitive CE method coupled with  $\text{Ru}(\text{bpy})_3^{2+}$ -ECL detection for the analysis of non-derivatized IBAN. The electrochemical and ECL behavior of IBAN and the optimal CE separation and ECL detection conditions were investigated. The applicability of this method to the analysis of IBAN in drug formulation and human urine was then examined. In order to remove the interference from urine matrix and to enrich the analyte, SPE using magnetic alumina-coated iron oxide nanoparticles ( $\text{Fe}_3\text{O}_4@/\text{Al}_2\text{O}_3$  NPs) as the extraction phase [13, 14] was employed to the pretreatment of urine sample.

Report on the use of magnetic SPE for the separation of drugs from biological matrices has been scarce [13]. Application of this technique to the extraction of phosphate-bearing drugs has not been reported either. We believe this is the first report on both direct CE-ECL analysis and magnetic SPE with  $\text{Fe}_3\text{O}_4@/\text{Al}_2\text{O}_3$  NPs for IBAN.

## 2 Materials and methods

### 2.1 Apparatus

Cyclic voltammetry (CV) and ECL studies were performed with a CHI model 635 electrochemical station (Austin, TX, USA). A three-electrode cell with an indium tin oxide (ITO) working electrode (Delta Technologies, Stillwater, MN, USA), an Ag/AgCl/saturated KCl reference and a Pt wire auxiliary electrode was used for electrochemical measurements. Optical signals were captured using a Hamamatsu R928 PMT (Hamamatsu City, Japan), positioned in front of the ITO electrode and biased at  $-900$  V.

The laboratory-assembled CE-ECL system was similar to that described previously [15]. Separation capillaries (Polymicro Technologies, Phoenix, AZ, USA) were of 50  $\mu$ m id  $\times$  360  $\mu$ m od  $\times$  30 cm length. Prior to use, the capillary was flushed sequentially with 0.5 M NaOH (10 min),  $\text{H}_2\text{O}$  (10 min) and electrophoretic buffer (15 min). Following each five injections, the capillary was rinsed with 0.1 M NaOH,  $\text{H}_2\text{O}$  and electrophoretic buffer for 5 min to maintain a reproducible inner surface. An ITO electrode, situated at the capillary outlet and biased at 1.6 V (versus a Pt-wire reference), was used for in situ generation of the active  $\text{Ru}(\text{bpy})_3^{3+}$ . Sample injection was carried out hydrodynamically for 20 s at 10 cm height.

### 2.2 Chemicals

IBAN sodium ( $\geq 97\%$ ) and tris(2,2'-bipyridyl)ruthenium(II) chloride ( $\text{Ru}(\text{bpy})_3\text{Cl}_2$ , 98%) were purchased from Sigma-Aldrich (Milwaukee, WI, USA). Sodium tripolyphosphate ( $\text{Na}_5\text{P}_3\text{O}_{10}$ ) was obtained from Showa (Tokyo, Japan). Iron oxide ( $\text{Fe}_3\text{O}_4$ , particle size 20–30 nm) and sodium silicate were bought from Alfa Aesar (Ward Hill, MA, USA). All other chemicals were of analytical-reagent grade. All solutions were filtered through a 0.45- $\mu$ m pore-size membrane filter before use.

### 2.3 Urine sample preparation

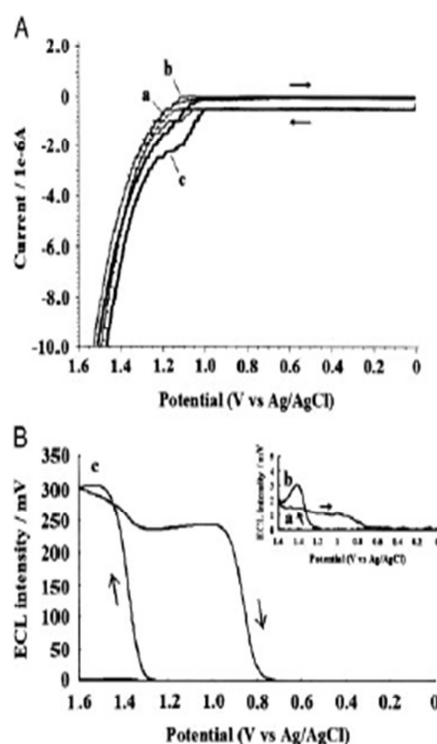
The fresh human urine sample of a healthy female volunteer was acquired from a student in the laboratory. Prior to analysis, 1.0 mL urine was added with 50  $\mu$ L of 0.1 M NaOH (as blank) or a standard solution of IBAN in 0.1 M NaOH (as sample) and 50  $\mu$ L of 1.0 M EDTA. The solution was thoroughly mixed, followed by ultrafiltration at

14 000 × g for 20 min using a microcentrifuge (model 3740; Kubota, Tokyo, Japan) coupled with Millipore's Amicon® Ultra-0.5 centrifugal filter devices (nominal molecular weight limit 3 kDa). The filtrate was then subjected to magnetic SPE using Fe<sub>3</sub>O<sub>4</sub>@Al<sub>2</sub>O<sub>3</sub> NPs as the solid phase. Preparation of the Fe<sub>3</sub>O<sub>4</sub>@Al<sub>2</sub>O<sub>3</sub> NPs has been described previously [14]. In a 2.0-mL polypropylene vial, 1.0 mL of the above filtrate was ultrasonically mixed with 1 mg of Fe<sub>3</sub>O<sub>4</sub>@Al<sub>2</sub>O<sub>3</sub> NPs for 5 min. The NPs that conjugated with IBAN were then aggregated by an external magnet, and the supernatant liquid was completely removed with a micropipette. The NPs were ultrasonically washed with 0.5 mL of 150 mM phosphate buffer solution (pH 9.0) for 5 min, and the supernatant liquid was discarded. This washing step was repeated twice more. Finally, IBAN on the NPs was rinsed off with 10 µL of 20 mM Na<sub>5</sub>P<sub>3</sub>O<sub>10</sub> solution (pH 9.0). The eluate was directly injected into the CE-ECL system for analysis. Due to the concern of possible contamination to the next sample, the used NPs were discarded without further cleaning.

### 3 Results and discussion

#### 3.1 Electrochemical and ECL behavior of IBAN on an ITO electrode

The electrochemical and Ru(bpy)<sub>3</sub><sup>2+</sup>-based ECL behavior of IBAN in aqueous solution was first studied using CV and an ITO electrode in a three-electrode cell. Figure 2A illustrates the cyclic voltammogram obtained in a 20-mM sodium phosphate buffer at pH 8.0 (curve a; dotted line). This background voltammogram is featureless in the potential range scanned (0–1.6 V). With the addition of 5 µM Ru(bpy)<sub>3</sub><sup>2+</sup>, the well-known reversible Ru<sup>2+/3+</sup> redox waves appear at ~1.1 V (curve b; thin line). In the presence of 0.15 mM IBAN, the anodic wave for Ru(bpy)<sub>3</sub><sup>2+</sup> oxidation at ~1.1 V is significantly enhanced (curve c; thick line). The catalytic current enhancement was found to be proportional to the concentration of IBAN in the solution. The redox waves of IBAN itself were not observed in the potential range scanned. The corresponding ECL-potential curves obtained during CV scan are illustrated in Fig. 2B. There is no ECL emission in the background electrolyte (curve a in inset). In the presence of 5 µM Ru(bpy)<sub>3</sub><sup>2+</sup>, weak ECL emission emerges following the oxidation of Ru(bpy)<sub>3</sub><sup>2+</sup> and reaches a maximum at ~1.4 V (curve b in inset). With the addition of 0.15 mM IBAN, significant enhancement (~100-fold) of ECL emission results (curve c), which evidences the electrocatalytic effect of IBAN on the Ru(bpy)<sub>3</sub><sup>2+</sup>-based ECL. Since IBAN is a tertiary amine, its electrochemical and ECL behavior in the Ru(bpy)<sub>3</sub><sup>2+</sup> solution should be similar to that of Ru(bpy)<sub>3</sub><sup>2+</sup>/tripropylamine system [16], and the mechanism of ECL reaction between Ru(bpy)<sub>3</sub><sup>2+</sup> and tertiary amine has been well-known [11]. Judging from the above results, Ru(bpy)<sub>3</sub><sup>2+</sup>-based ECL can be utilized as a sensitive tool for direct detection of IBAN.



**Figure 2.** (A) Cyclic voltammograms of 20 mM sodium phosphate, pH 8.0 (curve a), 5 µM Ru(bpy)<sub>3</sub><sup>2+</sup> in phosphate buffer (curve b) and 0.15 mM IBAN in phosphate buffer containing 5 µM Ru(bpy)<sub>3</sub><sup>2+</sup> (curve c). (B) Corresponding ECL-potential curves of (A). Conditions: working electrode, ITO (0.25 cm<sup>2</sup>); reference electrode, Ag/AgCl/saturated KCl; counter electrode, Pt wire; potential scan rate, 50 mV/s; PMT voltage, -900 V.

#### 3.2 Optimization of CE-ECL procedure

##### 3.2.1 Effect of detection potential

In the present work, a simple two-electrode cell with an ITO working electrode and a Pt wire pseudo-reference was used as the ECL detector for CE. The optimal detection potential was investigated by repeated injection of 10 µM IBAN standard into CE while varying the applied ITO electrode potential in the range 1.0–2.0 V. The ECL intensity started to increase from 1.2 V with increasing detection potential and reached a plateau at ~1.6 V (data not shown). The optimal detection potential for IBAN was therefore set at 1.6 V. This potential correlates well with the ECL result shown in curve c of Fig. 2B.

##### 3.2.2 Effect of Ru(bpy)<sub>3</sub><sup>2+</sup> concentration in the detection cell

The variation of ECL intensities on the concentrations of Ru(bpy)<sub>3</sub><sup>2+</sup> in the range of 1–5 mM was examined next. As



expected, the intensity of ECL emission increased with increasing  $\text{Ru}(\text{bpy})_3^{2+}$  concentration in the detection cell, but the background noise also increased with higher concentration of  $\text{Ru}(\text{bpy})_3^{2+}$ . The  $S/N$  ratio of ECL began to deteriorate after reaching the maximum at a  $\text{Ru}(\text{bpy})_3^{2+}$  concentration of 3.5 mM. Therefore, 3.5 mM  $\text{Ru}(\text{bpy})_3^{2+}$  was chosen as the optimum.

### 3.2.3 Effect of buffer pH

The effect of buffer pH on the CE of IBAN was examined in the pH range of 7.5–10. Using 20 mM sodium phosphate as the electrophoretic buffer, the optimal migration time and peak shape were found at pH 9.0. It is known that ECL intensity for the reaction of  $\text{Ru}(\text{bpy})_3^{2+}$  and tertiary amine is pH-dependent, and the optimal range is about pH 7–8 [17]. In the present study, we found that the optimum pH for ECL detection of IBAN was 8.0. Since the CE buffer (pH 9.0) in capillary continuously flowed into the detection cell during analysis, in order to offset the possible pH variation in the detector caused by CE eluate, a 200-mM sodium phosphate buffer at pH 8.0 was used in the ECL detection cell. This buffer solution was replaced with fresh one every 4 h to keep the pH value constant.

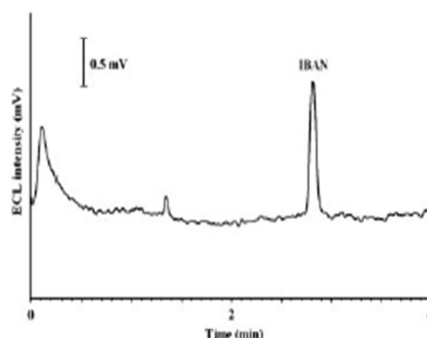
### 3.2.4 Effect of injection time

In the present study, sample injection was performed hydrodynamically by immersing the capillary inlet in the sample solution at 10 cm height relative to the outlet of the capillary for a fixed time period. The effect of injection time on the ECL intensity of IBAN peak was examined in the range of 5–40 s. The ECL intensity increased with increasing injection time. However, the efficiency, calculated as the number of theoretical plates,  $N$ , decreased with lengthy injection time. An injection time of 20 s was judged optimal, as a compromise between detection sensitivity and CE efficiency.

Figure 3 illustrates a typical electropherogram of IBAN obtained with ECL detection under the optimal experimental conditions described above. Sharp and symmetric peak could be observed. The number of theoretical plates,  $N$ , calculated from  $N = 5.55 \times (t_R/u_{0.5})^2$ , was  $1.05 \times 10^4$ . Complete elution of IBAN was achieved in about 2.8 min with a capillary of 30 cm length. There is an elution band appeared at about 0.2 min in Fig. 3. Although this band showed up in most of the electropherograms obtained in this work, its cause is not clear at present. The small peak emerged at 1.3 min is probably due to impure constituent in the commercial IBAN chemical (labeled purity  $\geq 97\%$ ) used for the preparation of standard solution.

## 3.3 Analytical performance characteristics

Calibration graph was constructed for IBAN in the concentration range 0.25–50  $\mu\text{M}$ . The linear regression equation was  $y = 2.14 (\pm 0.17) \times 10^3 x - 0.19 (\pm 0.02) \times 10^3$



**Figure 3.** Electropherogram of IBAN obtained with ECL detection. Conditions: capillary size, 50  $\mu\text{m}$  id  $\times$  360  $\mu\text{m}$  od  $\times$  30 cm length; anodic buffer, 20 mM sodium phosphate (pH 9.0); cathodic buffer, 200 mM sodium phosphate (pH 8.0) containing 3.5 mM  $\text{Ru}(\text{bpy})_3^{2+}$ ; CE voltage, 13.5 kV; hydrodynamic injection, 20 s at 10 cm height; ITO potential, 1.5 V; PMT voltage,  $-900$  V. IBAN concentration: 1  $\mu\text{M}$ .

**Table 1.** Intra-day and inter-day precisions of migration time and peak area for IBAN at different concentrations ( $n = 5$ )

Concentration ( $\mu\text{M}$ )	RSD (%)			
	Migration time		Peak area	
	Intra-day	Inter-day	Intra-day	Inter-day
0.5	0.5	0.7	5.1	7.3
2.5	0.6	0.7	3.9	5.8
20	0.4	0.8	3.1	4.4

( $r = 0.9992$ ,  $n = 7$ ), where  $y$  is the peak area and  $x$  is the concentration ( $\mu\text{M}$ ) of IBAN. The LOD ( $S/N = 3$ ) and LOQ ( $S/N = 10$ ) for IBAN in water were 0.08 and 0.25  $\mu\text{M}$ , respectively, calculated from the signal height (0.348 mV) of 0.25  $\mu\text{M}$  IBAN and the mean noise level (0.035 mV) at 2.8 min on the electropherograms of blank solution.

The intra-day precision of the method was examined by repeated injection of three standard IBAN solutions at low (0.5  $\mu\text{M}$ ), medium (2.5  $\mu\text{M}$ ) and high (20  $\mu\text{M}$ ) concentrations for five times on the same day. The inter-day precision was studied by analyzing the three standard samples one time each day for five consecutive days. The RSD values of the migration time and the ECL peak area are summarized in Table 1. The intra-day RSDs for migration time and peak area were  $< 0.6$  and 5.1%, respectively. The inter-day RSDs for migration time and peak area were  $< 0.8$  and 7.3%, respectively.

## 4 Applications

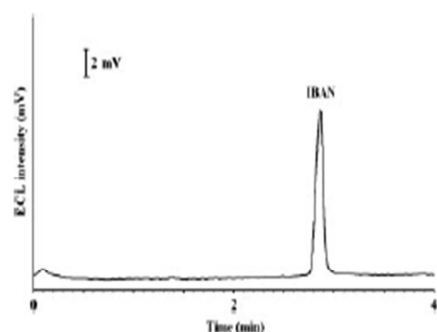
### 4.1 Analysis of drug formulation

The applicability of the developed CE-ECL method was first examined by analyzing an i.v. formulation of IBAN (Bonviva<sup>®</sup>)

from Roche (Switzerland). Owing to its relatively simple matrix, the only pretreatment performed was a 350-fold dilution with the CE buffer. Figure 4 shows the typical electropherogram obtained for IBAN in diluted Bonviva<sup>®</sup>. A single and sharp peak was observed. Based on triplicate analyses, the IBAN content in Bonviva<sup>®</sup> was found to be  $1003 \pm 75 \mu\text{g/mL}$  ( $n = 3$ ), which agrees well with manufacturer's certified value (3 mg ibandronic acid per 3 mL solution for i.v. injection).

#### 4.2 Analysis of human urine

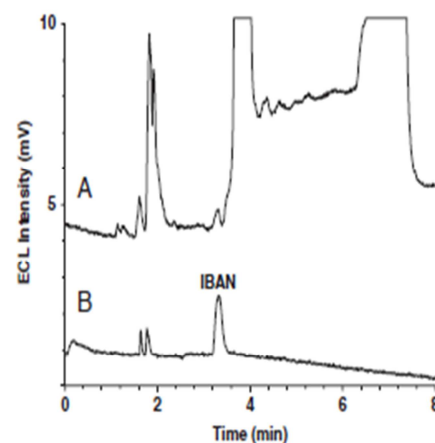
In order to assess the applicability of the method for biological or clinical analysis, IBAN-spiked human urine was used as the test sample. Due to the complex matrix of human urine, direct CE-ECL analysis of IBAN in urine was difficult. Tedious and time-consuming pretreatment of biological materials was always involved prior to IBAN analysis [4, 6, 7]. In this study, we attempted to develop a simple SPE procedure for IBAN in urine sample. SPE using magnetic  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs has been a popular technique for selective enrichment of phosphate-bearing biomolecules, e.g. phosphopeptides and phosphoproteins, from complex biological samples [18, 19]. We had also efficiently extracted a phosphate-bearing herbicide, glyphosate, from aqueous samples with SPE using  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs as the solid phase [14]. The high specificity is due to a bidentate binding nature between the phosphate group and metal oxide surface [20]. The paramagnetic properties of these particles also allow an easy isolation of products in solution by attracting them with the aid of an external magnetic field. In addition, magnetic NPs possess large surface area which facilitates SPE. Since IBAN is a bisphosphonate compound, a specific interaction between IBAN and  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs should also exist. A magnetic SPE procedure for IBAN in urine was therefore developed, as described in Section 2.3. This procedure was modified from our previous tactics of extracting the uniphosphonate herbicide, glyphosate, from aqueous sample with  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs [14]. Instead of using  $\text{Na}_4\text{P}_2\text{O}_7$  solution as the eluting agent IBAN attached on the magnetic NPs was rinsed off with  $\text{Na}_5\text{P}_3\text{O}_{10}$  solution. This modification was necessary because  $\text{P}_3\text{O}_{10}^{5-}$  revealed a higher eluting strength



**Figure 4.** Electropherogram of an intravenous formulation of IBAN (350-fold diluted Bonviva<sup>®</sup>). Conditions as in Fig. 3.

than  $\text{P}_2\text{O}_7^{3-}$  for IBAN. The optimal concentration of  $\text{Na}_5\text{P}_3\text{O}_{10}$  was found to be 20 mM. Figure 5(A) shows the typical electropherogram of a urine sample spiked with  $0.5 \mu\text{M}$  IBAN and subjected to ultrafiltration only. Despite the selectivity of  $\text{Ru}(\text{bpy})_3^{2+}$ -ECL detection, large extraneous peaks appear in the background, indicating that it is not possible to remove the interfering components in urine matrix by a single ultrafiltration pretreatment. Accurate determination of IBAN signal size would be difficult because the analyte peak situated very close to the huge extraneous peaks. Furthermore, the stability of CE capillary as well as the sensitivity of ITO electrode in the detection cell deteriorated rapidly with consecutive injections of urine sample, probably caused by fouling of the capillary wall and electrode surface. The electropherogram of the same urine sample subjected to ultrafiltration followed by SPE with  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs is illustrated in Fig. 5B. In comparison with Fig. 5A, those large matrix peaks totally disappear and just a single IBAN peak shows up. Besides, the peak size of IBAN in Fig. 5B is significantly larger than that in Fig. 5A, which evidences the efficiency and specificity of SPE with  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs for IBAN. The slight spreading of peak shape and longer migration time of IBAN observed in Fig. 5B, compared with Figs 3 and 4, may be due to conductivity and/or pH differences between injected analyte zone and CE background electrolyte [21].

Calibration graph was constructed using IBAN-spiked human urine in the concentration range of 0.2–12.0  $\mu\text{M}$ . The linear regression equation was  $y = 11.43(\pm 0.67) \times 10^3 x - 0.99(\pm 0.12) \times 10^3$  ( $r = 0.9974$ ,  $n = 6$ , where  $y$  is the peak area and  $x$  is the IBAN concentration in  $\mu\text{M}$ ). The LOD and LOQ for IBAN in urine were 0.06 and 0.2  $\mu\text{M}$  (19 and 64 ng/mL), respectively. As a comparison, Table 2 lists the reported LOD and/or LOQ values of IBAN in various sample matrices by different analytical methods. The LOD/LOQ values obtained with the developed CE-ECL method



**Figure 5.** Electropherograms of human urine sample containing  $0.5 \mu\text{M}$  IBAN. (A) Sample pretreatment with ultrafiltration only. (B) Sample pretreatment with ultrafiltration followed by SPE using magnetic  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs as the solid phase. Other conditions as in Fig. 3.

**Table 2.** Comparison of reported LOD/LOQ values of IBAN by various analytical methods

Method	Sample	LOD/LOQ (ng/mL)	Reference
GC-MS <sup>a)</sup>	Plasma	~1	4
	Urine	~2	
ELISA	Serum	~0.05	4
HPLC-MS/MS <sup>a)</sup>	Plasma	~0.2	7
HPLC-ELSD	Water	176 000	5
IC-conductometry	Drug	380 000/113 000	8
HPLC-fluorometry <sup>a)</sup>	Water	23	6
CE-ECL	Urine	19/64	This work

a) Derivatization required.

are more than three orders of magnitude lower than those of HPLC-ELSD [5] and IC conductometry [8] methods, and are similar to that of the technically complicated HPLC-photochemical derivatization-fluorometric detection method [6]. Although GC-MS [4] and HPLC-MS/MS [7] methods could provide lower ng/mL level LOQ for IBAN in biological fluids, both analyte derivatization and high-cost instrumentation were required. ELISA is the most sensitive and selective method for IBAN reported to date. However, its specific reagent is not readily available.

The intra-day and inter-day precisions of the method were evaluated using urine samples spiked with 0.5 and 5.0  $\mu$ M IBAN. The intra-day RSDs ( $n = 5$ ) for 0.5 and 5  $\mu$ M IBAN were 7.7 and 4.6%, respectively. The inter-day RSDs ( $n = 5$ ) for 0.5 and 5  $\mu$ M IBAN were 9.4 and 6.1%, respectively. Mean recoveries, calculated as the ratio of IBAN concentration found to that originally spiked, for 0.5 and 5  $\mu$ M IBAN were 102 and 97% ( $n = 5$ ), respectively. Total analysis time for each urine sample including pretreatment, SPE and CE-ECL was <1 h.

## 5 Concluding remarks

A simple, rapid and sensitive CE-ECL method for the analysis of IBAN has been developed. Compared with most chromatographic methods for IBAN reported to date, derivatization of the analyte prior to CE-ECL analysis is not necessary. High-cost instrumentation is also not required. The feasibility of efficient and accurate determination of IBAN in drug formulation by the new method was demonstrated. In couple with SPE pretreatment using magnetic  $\text{Fe}_3\text{O}_4/\text{Al}_2\text{O}_3$  NPs as solid phase, the proposed method was successfully applied to the determination of IBAN at sub- $\mu$ M level in human urine. The novel magnetic SPE procedure developed for IBAN in urine sample was simpler and faster than other reported pretreatment methods. Combining the selectivity of  $\text{Ru}(\text{bpy})_3^{2+}$ -ECL detection and the specificity of  $\text{Fe}_3\text{O}_4/\text{Al}_2\text{O}_3$  NPs extraction, this method seems particularly suitable for the analysis of phosphate-containing amines in biological and environmental

materials. To our knowledge, this is the first report on the development of both CE-ECL method and magnetic SPE pretreatment for trace IBAN in biological sample. Research on the development of CE-ECL method for other structurally similar bisphosphonate drugs is in progress.

Financial support from the National Science Council of Taiwan is gratefully acknowledged.

The authors have declared no conflict of interest.

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Received September 16, 2011  
Revised November 7, 2011  
Accepted December 1, 2011

## Research Article

# Analysis of alendronate in human urine and plasma by magnetic solid-phase extraction and capillary electrophoresis with fluorescence detection

A simple, rapid and sensitive CE-fluorescence (FL) detection method for the analysis of alendronate (ALEN), a bisphosphonate drug, has been developed. Using a buffer solution of 20 mM sodium phosphate (pH 10.0) and a voltage of 24 kV, separation of ALEN in a 55-cm length (35-cm effective length) capillary was achieved in 5 min. FL detection of ALEN was performed via pre-column derivatization with 2,3-naphthalene dicarboxaldehyde (NDA). Linear correlation ( $r = 0.9981$ ,  $n = 6$ ) between FL intensity and analyte concentration was obtained in the range of 7–200 ng/mL ALEN. The developed CE-FL method was applied to the analysis of ALEN in human urine and plasma samples. In order to eliminate the interfering matrix components, SPE using magnetic  $\text{Fe}_3\text{O}_4/\text{Al}_2\text{O}_3$  nanoparticles as solid sorbents was employed to clean the biological fluids before CE-FL analysis. The linear ranges of ALEN in urine and plasma were 5–100 ng/mL ( $r = 0.9982$ ,  $n = 7$ ) and 5–70 ng/mL ( $r = 0.9954$ ,  $n = 7$ ), respectively. The LOD and LOQ in both urine and plasma samples were 1.5 and 5 ng/mL ALEN, respectively. Total analysis time including sample pre-treatment and CE separation was less than 1.5 h.

**Keywords:** Alendronate / CE-fluorescence detection / Human urine / Magnetic SPE / Plasma  
DOI 10.1002/jscc.201100824

## 1 Introduction

Alendronate ((4-amino-1-hydroxybutylidene)bisphosphonate; ALEN) (Fig. 1) is a second generation amino-bisphosphonate drug widely used for the treatment of a variety of bone diseases including postmenopausal osteoporosis, Paget's disease and metastatic bone disease [1, 2]. Its primary mechanism of action involves the inhibition of osteoclastic bone resorption. The bioavailability of ALEN in human is low, only about 0.75% [3]. Besides, it does not undergo metabolic biotransformation in human [4]. Following oral or intravenous administration, ALEN is either sequestered in bone or excreted unchanged in the urine. Since urinary excretion is the sole means of ALEN elimination, renal clearance measurements provide the most useful approach for bioequivalence studies [5].

Analysis of ALEN, and other bisphosphonate drugs as well, often represents a challenge because of their polar ionic nature and chelation properties; they may interact with metals in HPLC systems resulting in poor peak shape and irreproducible chromatography. In addition, ALEN possesses no chromophore or fluorophore, complexation with metal ions or derivatization with fluorescent dyes prior to UV or fluorescence (FL) detection is necessary. Furthermore, bisphosphonates are not typically amenable to analysis by HPLC with MS/MS detection because they produce a distribution of multiply charged ions and are prone to adduct formation under ESI conditions; these factors limit assay sensitivity [6]. A review of analytical methods for the determination of various bisphosphonates, including ALEN, in pharmaceutical and biological material has been published recently [7]. In general, RP HPLC-FL methods with pre-column derivatization using 2,3-naphthalene dicarboxaldehyde (NDA) [8, 9], 9-fluorenylmethyl chloroformate (FMOCl) [10–12], *o*-phthalaldehyde (OPA) [13] and fluorescamine [14] have been the most popular for the determination of ALEN in drug formulation, plasma and urine samples. Ion chromatography (IC) coupled with non-derivatization detection methods, such as indirect UV detection [15, 16], conductivity detection [17], refractive index detection [18] and inductively coupled plasma (ICP)-MS detection [19] also have been employed to the

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**Abbreviations:** ALEN, alendronate;  $\text{Fe}_3\text{O}_4/\text{Al}_2\text{O}_3$  NPs, alumina-coated iron oxide nanoparticles; NDA, 2,3-naphthalene dicarboxaldehyde

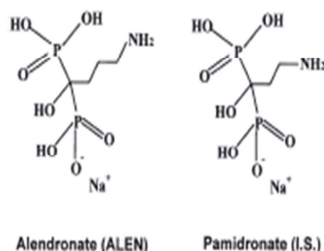


Figure 1. Structural formula of alendronate sodium and pamidronate sodium.

analysis of ALEN in pharmaceutical and biological samples. Derivatization with isobutyl chloroformate followed by HPLC-MS analysis has been reported for ALEN in human urine [20]. Derivatization with diazomethane followed by HPLC-MS/MS analysis also has been developed for ALEN in human serum and urine [21]. LOQs of ALEN in serum and urine were 0.05 and 1 ng/mL, respectively, which were the lowest LOQs reported to date.

CE has been a popular technique for drug analysis because of its high efficiency and versatility, very low sample and reagent consumption, low cost and minimization of environmental pollution. However, the number of literature related to the use of CE for ALEN is limited, which may be attributed to the lack of sensitive detection method. A CE-UV procedure for the determination of ALEN in dosage form was described [22]. Direct UV detection was based on the online formation of a chromophoric complex between ALEN and Cu(II) present in the BGE. LOQ was in the  $\mu\text{g/mL}$  level. Several amino-bisphosphonate pharmaceuticals including ALEN in dosage form and human urine have been determined by multidimensional CE techniques [5]. CITP was employed as a pre-separation and online sample concentration step prior to analytical determination using either CITP or CZE coupled with conductivity detection. LOQs of ALEN in urine were about 3–10 ng/mL. Recently, a rapid CE-indirect UV method for alkylphosphonate drugs including ALEN in dosage form was reported [23]. Indirect UV detection of those non-chromophore drugs was based on the use of multiple probes BGE. The main drawback of this method was its relatively poor LOD ( $50 \mu\text{g/mL}$ ) of ALEN. From the viewpoint of drug analysis, CE assays with LOQs < 10 ng/mL are required to support pharmacokinetic studies because of the low bioavailability of ALEN and its low ng/mL levels in urine or plasma at therapeutic doses.

Matrix interference has always been a problem during analysis of ALEN in biological samples. According to the literature, a sequence of sample pre-treatment steps is generally involved prior to instrumental analysis. Precipitation of ALEN as calcium salt in alkaline medium, followed by acid dissolution of the precipitate and SPE purification of the analyte, has been an efficient and widely applied protocol [8, 9, 12, 13]. However, the procedure was tedious and time-consuming. Recently, SPE using magnetic materials as solid sorbents has become a rapid and versatile

technique for sample treatment and analyte pre-concentration [24]. Magnetic  $\text{Fe}_3\text{O}_4$  coated with  $\text{Al}_2\text{O}_3$  or  $\text{TiO}_2$  core-shell nanoparticles have been particularly employed to the selective enrichment of phosphate-bearing biomolecules, e.g. phosphopeptides and phosphoproteins, from complex biological samples [25, 26]. The high specificity is due to a bidentate-binding nature between the phosphate group and metal oxide surface. The paramagnetic properties of these particles also allow an easy isolation of products in solution by attracting them with the aid of an external magnetic field. Furthermore, magnetic NPs possess large total surface area that facilitates SPE operation.

In this paper, we describe a simple, rapid and sensitive CE-FL method for analysis of ALEN in human urine and plasma. Pre-column derivatization of ALEN was performed using NDA, followed by CE with FL detection. In order to suppress interference from biological sample matrix and to enhance selectivity, magnetic SPE using alumina-coated iron oxide nanoparticles ( $\text{Fe}_3\text{O}_4@/\text{Al}_2\text{O}_3$  NPs) as extraction phase was employed to the pre-treatment of urine and plasma samples. Report on the use of magnetic SPE for the separation of drugs from biological matrices has been scarce [24]. Recently, we have demonstrated the successful use of  $\text{Fe}_3\text{O}_4@/\text{Al}_2\text{O}_3$  NPs as a selective sorbent to extract ibandronate, also an amino-bisphosphonate drug, from human urine [27]. In this work, we attempt to employ this magnetic SPE protocol to ALEN in urine and plasma samples. To our knowledge, this is the first report on both CE-FL analysis and magnetic SPE of ALEN drug.

## 2 Materials and methods

### 2.1 Apparatus

The CE-FL system was assembled in laboratory. A 0–30-kV power supply (Glassman High Voltage, Whitehouse Station, NJ, USA) provided the separation voltage. Fused-silica capillaries ( $50 \mu\text{m}$  id  $\times$   $365 \mu\text{m}$  od  $\times$   $55 \text{ cm}$  total length, Polymicro Technologies, Phoenix, AZ, USA) were used for separation. Prior to use, the capillary was flushed sequentially with 0.5 M NaOH (10 min),  $\text{H}_2\text{O}$  (10 min) and electrophoretic buffer (15 min). Following each five injections, the capillary was rinsed with 0.1 M NaOH,  $\text{H}_2\text{O}$  and electrophoretic buffer for 5 min to maintain a reproducible inner surface. FL detection was performed with an Argos 250B fluorescence detector (Flux Instruments AG, Basel, Switzerland). The detector consisted of a CE flow-cell module, special collection optics, optical fiber conduit, filter holder and a photomultiplier tube (PMT). A 75-W Xe-Hg arc lamp was used as the excitation source. The excitation wavelength was centered at 420 nm using an interference filter. The fluorescence emission was collected with a 495-nm cut-off filter. The PMT was operated at  $-950 \text{ V}$ . Sample injection was generally carried out in hydrodynamic mode for a fixed time period at 20 cm height, unless stated otherwise.

## 2.2 Chemicals and solutions

Alendronate sodium, pamidronate disodium (as internal standard; I.S.) and aluminum isopropoxide were purchased from Sigma-Aldrich (Milwaukee, WI, USA). NDA was obtained from Fluka (Buchs, Switzerland). Potassium cyanide was acquired from E. Merck (Darmstadt, Germany). Sodium tripolyphosphate ( $\text{Na}_5\text{P}_3\text{O}_{10}$ ) was obtained from Showa (Tokyo, Japan). Iron oxide ( $\text{Fe}_3\text{O}_4$ , particle size 20–30 nm) and sodium silicate were obtained from Alfa Aesar (Ward Hill, MA, USA). All other chemicals were of analytical-reagent grade.

Stock solutions of ALEN and I.S. at 1 mg/mL were prepared in distilled-deionized water. NDA solution of 10 mM was prepared in HPLC-grade methanol. KCN solution of 10 mM was prepared in 20 mM  $\text{Na}_2\text{HPO}_4$  solution (pH 10.0). All solutions were filtered through a 0.45- $\mu\text{m}$  pore-size membrane filter before use.

## 2.3 Preparation of urine sample

The fresh human urine sample of a healthy female volunteer was acquired from a student in the laboratory. Preparation of urine sample was similar to that described previously [27]. In brief, 1.0 mL urine was added with 50  $\mu\text{L}$  of 0.1 M NaOH (as blank) or a standard solution of ALEN in 0.1 M NaOH (as sample) and 50  $\mu\text{L}$  of 1.0 M EDTA. The solution was thoroughly mixed, followed by ultrafiltration at  $14\,000 \times g$  for 20 min using a microcentrifuge (model 3740; Kubota, Tokyo, Japan) coupled with Millipore's Amicon® Ultra-0.5 centrifugal filter devices (nominal molecular weight limit (NMWL) 3 kDa). The filtrate was then subjected to magnetic SPE using  $\text{Fe}_3\text{O}_4@/\text{Al}_2\text{O}_3$  NPs as the solid phase.  $\text{Fe}_3\text{O}_4@/\text{Al}_2\text{O}_3$  NPs were prepared following the procedure depicted in the literature [28].

## 2.4 Preparation of plasma sample

Blood samples placed in freeze-dried EDTA tubes were centrifuged immediately to obtain the plasma, which was stored  $-20^\circ\text{C}$  until analysis. To 400  $\mu\text{L}$  of plasma sample, 50  $\mu\text{L}$  of ALEN standard solution was added. The plasma mixture was deproteinized by adding 450  $\mu\text{L}$  of acetonitrile. After centrifugation at  $20\,000 \times g$  for 15 min, the supernatant liquid was subjected to magnetic SPE treatment described below.

## 2.5 Magnetic SPE procedure

In a 2.0-mL polypropylene vial, 1.0 mL of the above urine filtrate or 0.8 mL of the above plasma filtrate was ultrasonically mixed with 1 mg of  $\text{Fe}_3\text{O}_4@/\text{Al}_2\text{O}_3$  NPs for 5 min. The NPs that conjugated with ALEN were then aggregated by an external magnet, and the supernatant

liquid was completely removed with a micropipette. The NPs were ultrasonically washed with 0.5 mL of 150 mM phosphate buffer solution (pH 9.0) for 5 min, and the supernatant liquid was discarded. This washing step was repeated twice. Finally, ALEN on the NPs was rinsed off with 10  $\mu\text{L}$  of 20 mM  $\text{Na}_5\text{P}_3\text{O}_{10}$  solution (pH 9.0). The eluate was then subjected to derivatization treatment as described below. Due to concern of possible contamination to the next sample, the used NPs were discarded without further cleaning.

## 2.6 Derivatization protocol

The NDA-derivatization procedure followed that reported by Roach and Harmony [29]. Optimized derivatization parameters, e.g. NDA concentration, reaction pH, time and temperature, were adopted from our previous work [30]. In a 1.0-mL polypropylene vial, 10  $\mu\text{L}$  of ALEN standard or the above eluate were mixed with 5  $\mu\text{L}$  of 1  $\mu\text{g}/\text{mL}$  pamidronate (I.S.) and 1.5  $\mu\text{L}$  of 10 mM KCN solution (pH 10.0). A 1.5- $\mu\text{L}$  aliquot of 10 mM NDA solution was then added. The reaction vial was covered with aluminum foil to prevent photochemical decomposition of the product and the solution mixture was thoroughly mixed at room temperature for 20 min using a vibrating mixer. The resulting solution was directly injected into the CE-FL system for analysis. All ALEN concentrations reported in this work were the initial concentration of ALEN in the sample solution prior to treatment and derivatization.

## 3 Results and discussion

### 3.1 Study of CE-FL conditions for ALEN

NDA reacts with primary amines in the presence of  $\text{CN}^-$  to form 1-cyanobenzofisoindole derivatives, which possess excellent thermal stability and high quantum yields [29]. In addition to an intense absorption band in the UV region at approximately 250 nm, the derivatives exhibit two weaker excitation maxima in the visible region at approximately 420 and 440 nm with fluorescence maximum at 490 nm. In this study, FL detection of the NDA-derivatized ALEN and I.S. was performed with an excitation wavelength of 420 nm and emission light collected at wavelength  $>495$  nm. Pamidronate (Fig. 1), a first generation bisphosphonate drug structurally similar to ALEN, was selected as the I.S. Effect of BGE concentrations (sodium phosphate buffer, pH 10.0) on the separation of the two NDA-labeled bisphosphonate drugs was examined first. Figure 2A shows the variation of mobilities of ALEN and I.S. in the BGE concentration range 10–40 mM. Separation between the two NDA derivatives improved with increasing BGE concentrations. A resolution ( $R_s$ ) of 1.24, calculated as  $2(t_{1,S} - t_A)/(w_{1,S} + w_A)$ , where  $t_{1,S}$ ,  $t_A$  and  $w_{1,S}$ ,  $w_A$  are the migration times and peak widths of the two derivatives, respectively, can be achieved with a BGE

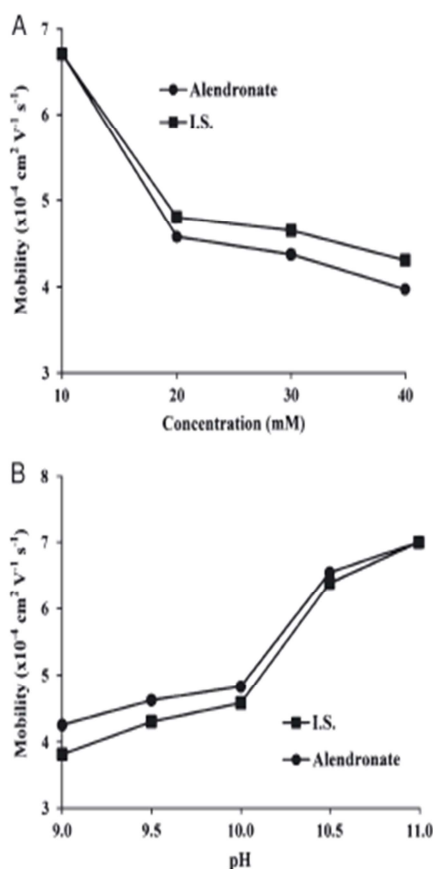


Figure 2. Effect of (A) BGE concentration and (B) BGE pH on the mobilities of NDA-labeled ALEN and I.S.

concentration of 20 mM. Higher concentrations led to larger  $R_s$  values, but with longer migration times. Therefore, 20 mM sodium phosphate buffer was chosen as the optimal.

Both ALEN and I.S. are amphoteric compounds with multiple  $pK_a$  values. After derivatization, the NDA-labeled ALEN and I.S. mainly exist as anions in aqueous BGE. Their charges as well as electrophoretic mobilities will depend upon the pH of BGE. Effect of pH on the mobilities of the two bisphosphonate derivatives was studied in the pH range 9.0–11.0 using 20 mM sodium phosphate buffer as BGE. The results are shown in Fig. 2B. Separation of the two derivatives was better at lower pH, but gradually deteriorated with increasing pH. This was probably caused by an increase in EOF at higher pH values of the BGE, which reduced the resolution of the given peak pair under counter-electroosmotic CE mode [31]. Poor resolution ( $R_s < 1.0$ ) was observed at pH 10.5 and beyond, and optimal separation of the two peaks can be obtained at pH 10.0. In order to achieve the objective of rapid separation and optimal resolution, the pH of the BGE was selected at 10.0. However, due to its relatively poor buffer capacity at this pH, unstable

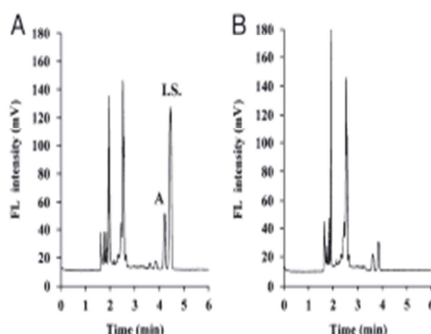


Figure 3. Electropherograms of ALEN and I.S. in water with FL detection. (A) NDA-labeled ALEN (peak A) and pamidronate (peak I.S.); (B) blank. Conditions: capillary,  $50 \mu\text{m}$  id  $\times$   $365 \mu\text{m}$  od  $\times$  55 cm length (35 cm to the detector); BGE, 20 mM sodium phosphate (pH 10.0); separation voltage, 24 kV; hydrodynamic injection, 10 s at 20 cm height; FL detection,  $\lambda_{\text{exc}}$ : 420 nm,  $\lambda_{\text{em}}$ :  $> 495$  nm; PMT voltage,  $-950$  V. Analyte concentration, ALEN, 100 ng/mL; I.S., 280 ng/mL.

migration times were sometimes observed. On the other hand, the relative migration times of ALEN/I.S. were found fairly constant, which could be attributed to the proper use of I.S. The BGE solution of CE was replaced with fresh one every 1–2 h in order to keep the pH value constant and the absolute migration times stable.

A typical electropherogram of NDA-labeled ALEN and I.S. is shown in Fig. 3. Sharp and symmetric peaks for the two NDA derivatives were obtained, with an average theoretical plate number of ca. 14 000. Under a capillary length of 55 cm (effective length 35 cm) and a separation voltage of 24 kV, optimal separation ( $R_s = 1.27$ ) of ALEN and I.S. was achieved in less than 5 min. The extraneous peaks appeared in Fig. 3A before 3 min and the two small peaks at 3.5–4.0 min may be due to reaction products between the excess derivatizing agent and the impurities in background solvent, as shown by the blank electropherogram (Fig. 3B).

A calibration curve for ALEN was constructed over the concentration range 7–200 ng/mL. The ratio of peak area of ALEN/I.S. versus concentration plot showed a good linearity ( $y = 2.6 \times 10^{-3}x + 6.4 \times 10^{-3}$ ) with a correlation coefficient ( $r$ ) of 0.9981 ( $n = 6$ ). At a concentration level of 50 ng/mL ALEN, the RSD values on the ratios of migration time and peak area of ALEN/I.S. were 0.53 and 2.31%, respectively. The LOD ( $S/N = 3$ ) and LOQ ( $S/N = 10$ ) values for ALEN in aqueous solution were calculated to be 2 and 7 ng/mL, respectively.

### 3.2 Analysis of human urine

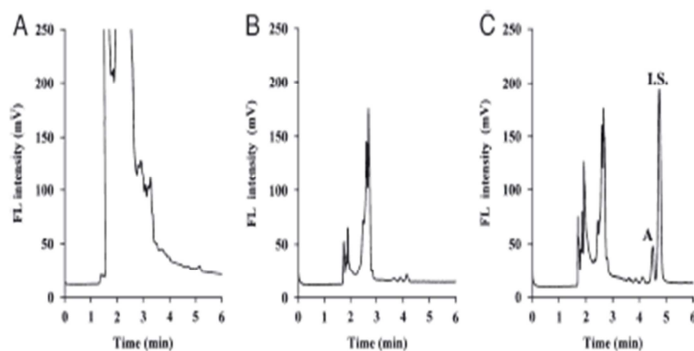
Despite that low ng/mL level of LOD and LOQ were readily achievable with CE-FL method, direct analysis of ALEN in urine sample was problematic due to complex matrix of human urine. In addition, the derivatization reaction

between ALEN and NDA may be affected by those endogenous urine components because NDA can react with other primary amines and amino acids as well. Recently, we had developed a magnetic SPE procedure for ibandronate, a bisphosphonate drug, in human urine using  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs as solid sorbents [27]. In this study, we attempted to use a similar extraction strategy for ALEN in urine sample. The procedure was described in Section 2.5. Figure 4 demonstrates the efficacy of magnetic SPE for ALEN in human urine. The electropherogram of a blank urine sample subjected to ultrafiltration only but without magnetic SPE treatment is shown in Fig. 4(A). The huge extraneous peaks in the background were caused by the endogenous urine matrix, indicating that it is impossible to remove the interfering components in urine matrix by a single ultrafiltration pre-treatment (see Section 2.3). Analysis of ALEN would be difficult because the analyte signal situated very close to the large extraneous peaks. In addition, the stability of CE capillary deteriorated rapidly with consecutive injections of urine sample, probably due to fouling of the capillary wall. The electropherogram of the same urine sample subjected to ultrafiltration and followed by magnetic SPE with  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs as the solid phase is illustrated in Fig. 4B. In comparison with Fig. 4A, the matrix interference was clearly removed and a flat baseline at the migration time region (ca. 4.5–5 min) of ALEN and I.S. was observed. The cleanness of the background was attributed to

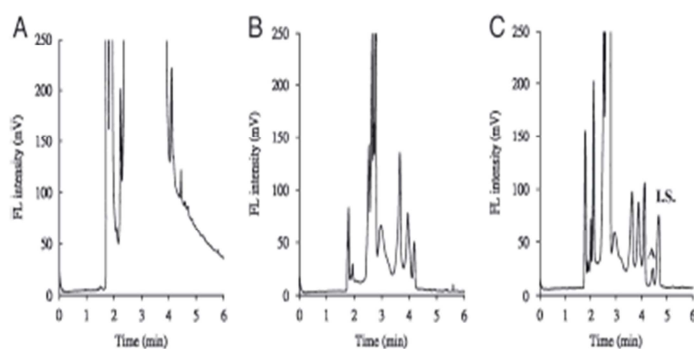
the selectivity of  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs and the efficiency of the triplicate washing steps with 150 mM phosphate buffer (pH 9.0) prior to the final desorption step. Without an appropriate washing, such a flat baseline could not be achieved. The electropherogram of the same urine sample spiked with 30 ng/mL ALEN and subjected to ultrafiltration and magnetic SPE treatment is shown in Fig. 4C. Sharp and significant peaks for the NDA-labeled ALEN and I.S. were readily obtained. No interference from the urine matrix was found, which evidenced the high efficiency and specificity of the magnetic SPE for ALEN in urine. Total sample treatment time including extraction and derivatization was about 1 h, and CE analysis time was 5 min.

### 3.3 Analysis of plasma

The developed method was also applied to analyzing ALEN in human plasma. Due to its high protein content, deproteinization of plasma sample prior to analysis was required. Deproteinization by ultrafiltration was initially attempted, but the centrifugal filter device (NMWL 3 kDa) was easily clogged. Deproteinization using acetonitrile in a 1:1 v/v ratio was alternatively carried out prior to magnetic SPE and derivatization treatment. Figure 5 shows the results of plasma analysis. The electropherogram of a deproteinized plasma sample is illustrated in Fig. 5A.



**Figure 4.** Electropherograms of ALEN in urine with FL detection. (A) Blank urine subjected to ultrafiltration only; (B) blank urine subjected to ultrafiltration and magnetic SPE; (C) urine spiked with 30 ng/mL ALEN followed by ultrafiltration and magnetic SPE. Conditions as in Fig. 3 except hydrodynamic injection time 15 s.



**Figure 5.** Electropherograms of ALEN in plasma with FL detection. (A) Blank plasma subjected to deproteinization only; (B) blank plasma subjected to both deproteinization and magnetic SPE; (C) plasma spiked with 10 ng/mL ALEN followed by deproteinization and magnetic SPE. Conditions as in Fig. 3, except that the concentration of I.S. added was 60 ng/mL and the electrokinetic injection time was 2 s at 21 kV.



The huge extraneous peaks were caused by the endogenous plasma components, which may include various amino acids and peptides. Obviously, a single deproteinization treatment was insufficient to remove those small amino compounds in plasma. It would not be possible to analyze trace ALEN in deproteinized plasma sample without further purification. The electropherogram of a deproteinized plasma sample also subjected to magnetic SPE using  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs as solid sorbents is shown in Fig. 5B. In comparison to Fig. 5A, most interfering components in plasma matrix were removed and a flat baseline appeared at the region of expected migration times of analytes, which again proved the efficiency of magnetic SPE treatment. The electropherogram of a plasma sample spiked with 10 ng/mL ALEN followed by deproteinization and magnetic SPE is shown in Fig. 5C. Despite the relatively complex background, both ALEN and I.S. peaks could be readily identified without interference. It is noted that in order to maintain an optimal resolution, the concentration of I.S. contained in the final sample solution was lowered to 60 ng/mL and injection was performed electrokinetically at 21 kV for 2 s.

### 3.4 Analytical figures of merit

Calibration graphs were constructed using ALEN-spiked human urine and plasma in the concentration ranges of 5–100 ng/mL (for urine) and 5–70 ng/mL (for plasma). The two linear regression equations were  $y = 4.1 \times 10^{-3}x - 4.6 \times 10^{-3}$  ( $r = 0.9982$ ,  $n = 7$ ) for urine and  $y = 2.1 \times 10^{-2}x + 1.7 \times 10^{-2}$  ( $r = 0.9954$ ,  $n = 7$ ) for plasma, where  $y$  is the ratio of peak area of ALEN/I.S. and  $x$  is the concentration of ALEN in ng/mL. The LOD ( $S/N = 3$ ) and LOQ ( $S/N = 10$ ) for ALEN in both urine and plasma were 1.5 and 5 ng/mL, respectively. These LOD/LOQ values were more than three orders of magnitude lower than those reported using CE with direct [22] or indirect [23] UV detection, and were in the same order of magnitude as those reported using multidimensional CE-conductivity detection [5] for urine analysis. In comparison to HPLC methods coupled with FL detection [8, 9], electrochemical detection [9], MS [20] and MS/MS [21] detection in plasma and urine analyses, the developed CE-FL method had a similar analytical sensitivity but simpler sample preparation procedure.

Within-day and between-day recovery and precision of the method were evaluated using urine and plasma samples spiked with low (10 ng/mL), medium (30 ng/mL) and high (80 ng/mL for urine and 50 ng/mL for plasma) concentrations of ALEN. The results of this analysis are illustrated in Table 1. Within-day and between-day recoveries for the three spiked levels of ALEN in urine are in the ranges 101–115 and 99–109%, respectively. Within-day and between-day precisions, represented as RSD, for the three spiked levels of ALEN are in the ranges 1.7–5.5 and 3.5–7.6%, respectively. Within-day and between-day recoveries of ALEN in plasma

**Table 1.** Within-day and between-day recovery and precision for CE-FL analysis of alendronate in human urine and plasma ( $n = 5$ )

Nominal concentration (ng/mL)	Within-day		Between-day	
	Recovery (%) <sup>a)</sup>	Precision (%) <sup>b)</sup>	Recovery (%)	Precision (%)
<i>Urine</i>				
10	115	5.5	109	7.6
30	102	3.6	101	4.6
80	101	1.7	99	3.5
<i>Plasma</i>				
10	105	7.8	112	10.3
30	98	5.1	96	8.1
50	97	4.1	101	5.4

a) Recovery expressed as [(mean observed concentration)/(nominal concentration)]  $\times$  100.

b) Precision expressed as RSD.

are in the ranges 97–105 and 96–112%, respectively. Within-day and between-day precisions of ALEN in plasma are in the ranges 4.1–7.8 and 5.4–10.3%, respectively. These values meet the criteria of accuracy and precision set in the bioanalytical method validation guidance [32].

Intermediate precision, defined as the long-term variability of the measurement process within a single laboratory over a number of weeks [33], was also examined. Plasma samples spiked with 10, 30 and 50 ng/mL ALEN were each analyzed six times at different days randomly within a span of three weeks. The recoveries were 96, 98 and 102%, and the RSD values were 8.6, 5.6 and 3.5 for 10, 30 and 50 ng/mL ALEN, respectively. The stability of the method could be attributed to the proper selection of pamidronate as the I.S., which is structurally very similar to ALEN. We did observe some changes on the absolute migration times and peak areas of analytes in electropherograms acquired at different days, but the relative migration times and peak areas of ALEN/I.S. were always constant. Therefore, the use of an appropriate I.S. is important in quantitative works with CE, particularly for analyzing biological samples.

### 4 Concluding remarks

A simple, rapid and sensitive CE-FL method for the analysis of ALEN in biological fluids has been developed. In couple with SPE pre-treatment using magnetic  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs as the solid phase, the proposed method was successfully applied for the determination of ALEN at low ng/mL level in human urine and plasma. The magnetic SPE procedure developed for ALEN in urine and plasma was simpler and faster than other reported sample pre-treatment strategies. Combining the efficiency of CE separation, the sensitivity of FL detection and the specificity of magnetic SPE using  $\text{Fe}_3\text{O}_4@Al_2O_3$  NPs, this method seems particularly suitable

for the analysis of phosphate-bearing amino drugs in biological materials. We trust that this is the first report on the development of both CE-FL method and magnetic SPE pre-treatment for trace ALEN in biological samples. Application of this method to other structurally similar bisphosphonate drugs is in progress in our laboratory.

Financial support from the National Science Council of Taiwan is gratefully acknowledged.

The authors have declared no conflict of interest.

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## A simple and rapid screening method for glyphosate in water using flow-injection with electrochemiluminescence detection

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Cite this: *Anal. Methods*, 2013, 5, 6186Received 27th June 2013  
Accepted 1st September 2013  
DOI: 10.1039/c3ay41059e  
[www.rsc.org/methods](http://www.rsc.org/methods)

A flow-injection (FI) system coupled with the electrochemiluminescence (ECL) detection method for rapid screening of glyphosate (GLY) in water has been developed. A wall-jet type ECL detector coupled with an indium/tin oxide (ITO)-coated glass working electrode was modified from a thin-layer electrochemical cell, and tris(2,2'-bipyridyl)ruthenium(II) ( $\text{Ru}(\text{bpy})_3^{2+}$ )-based ECL reaction was used for the sensitive detection of GLY. Under optimized conditions, the linear range of the method was 0.03–2.81  $\text{mg L}^{-1}$  GLY with  $R^2 = 0.9998$  ( $n = 7$ ). Repeatability was 5.3% ( $n = 27$ ) and the detection limit ( $3\sigma$ ) was 0.03  $\text{mg L}^{-1}$  GLY. Sample throughput was about 100 injections  $\text{h}^{-1}$ . Possible interference from some multivalent cations can be readily eliminated by passing the acidified water sample through a solid-phase extraction column packed with strong cation-exchange materials before analysis. The method was examined by application to surface waters including tap water and irrigation water.

### 1. Introduction

Glyphosate (*N*-(phosphonomethyl)glycine; GLY) is a broad-spectrum herbicide widely used to kill unwanted plants both in agricultural and in nonagricultural landscapes. Due to its relatively low mammal toxicity, GLY has become the most extensively used herbicide worldwide. However, GLY resistance in weeds has evolved under current GLY usage, with little or no diversity in weed management practices.<sup>1</sup> Besides, its indiscriminate application also generates some concerns regarding the possible health hazard and environmental contamination caused by GLY.<sup>2,3</sup> In order to protect human health, various countries have set up maximum residual limits of GLY in water and crops. The US Environmental Protection Agency (EPA),<sup>4</sup> Health Canada,<sup>5</sup> and China's Ministry of Water Resources<sup>6</sup> have set the maximum acceptable concentration (MAC) of GLY in drinking water at 0.7, 0.28 and 0.7  $\text{mg L}^{-1}$ , respectively. In Taiwan, the MAC of GLY in effluent was set at 1.0  $\text{mg L}^{-1}$ .<sup>7</sup> In this regard, there is a growing need to develop a rapid and sensitive method for monitoring GLY at residue levels in environmental waters.

Various methods have been reported for quantitative analysis of GLY in water, environmental matrices and agricultural products.<sup>8</sup> It is known that GLY often represents an analytical challenge because of its high water solubility and organic solvent insolubility, which make the extraction and pre-concentration of GLY from sample matrices difficult. Moreover, since GLY possesses no chromophore or fluorophore in its

molecular structure, both pre- and post-column derivatizations have therefore been employed to GC,<sup>9,10</sup> HPLC<sup>11,12</sup> and CE<sup>13,14</sup> of GLY followed by spectrophotometric detection. However, the procedure of derivatization is always tedious and time-consuming, and sometimes generates unstable products. Non-derivatization methods, such as indirect UV detection,<sup>15</sup> indirect fluorescence detection,<sup>16</sup> electrospray ionization-MS detection,<sup>17</sup> amperometric detection,<sup>18</sup> coulometric detection,<sup>19</sup> contactless conductivity detection,<sup>20</sup> electrochemiluminescence (ECL) detection,<sup>21</sup> and inductively coupled plasma (ICP)-MS detection<sup>22</sup> have also been coupled to HPLC or CE for GLY analyses. These methods often suffer from drawbacks such as inadequate sensitivity and the requirement of costly apparatus. In addition, due to the inclusion of a chromatographic or an electrophoretic separation process in the method, the speed of analysis, and therefore sample throughput, is always low. The commercial immunoassay kit for GLY can provide fast, selective and highly sensitive screening analysis,<sup>23</sup> but it is highly expensive. As a screening method, it should be able to select from an initial set of samples, those that are above a pre-set concentration level in a simple, fast, economic and reliable way. Only those samples that provide a positive response need to be further processed with a more sophisticated and expensive analytical method.

Flow injection (FI)<sup>24</sup> analysis is a simple, rapid, and versatile technique that is now firmly established, with widespread application mainly in environmental and clinical areas. Determination of GLY by the FI method is not common. Adcock *et al.*<sup>25</sup> reported a FI-chemiluminescence (CL) method for the analysis of GLY in industrial and commercial formulations. The active CL reagent, tris(2,2'-bipyridyl)ruthenium(II) ( $\text{Ru}(\text{bpy})_3^{2+}$ ),

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was chemically generated using a lead dioxide recirculating system. Colombo and Masini<sup>26</sup> developed a sequential injection method for the fluorometric determination of GLY based on the adaptation of the post-column derivatization reaction between glycine and *o*-phthalaldehyde in the presence of 2-mercaptoethanol, applicable to soil and sediment samples. Recently, Silva *et al.*<sup>27</sup> published a screening method for the determination of GLY in water samples using a multi-pumping flow system. The method was based on the reaction between GLY and *p*-dimethylaminocinnamaldehyde in an acid medium where the reaction product can be measured spectrophotometrically. In comparison with chromatography- or electrophoresis-based methods, FI is advantageous as a screening method due to its high sample throughput, simple apparatus and versatile detection schemes.

In this paper, we describe a simple, rapid, and sensitive screening method for GLY in water samples using FI coupled with Ru(bpy)<sub>3</sub><sup>2+</sup>-based ECL detection. This detection strategy has been successfully employed to HPLC<sup>28</sup> and CE<sup>23,29</sup> of GLY. The applicability of the developed FI-ECL method was examined by analyzing GLY in surface water samples.

## 2. Experimental

### 2.1. Apparatus

The FI-ECL detection system was assembled in the laboratory. The FI system consisted of a Rainim model RP-1 peristaltic pump (Emeryville, CA, USA), a Rheodyne model 9125 injection valve with 10  $\mu$ L sample loop, an interconnecting Teflon tubing (250  $\mu$ m i.d.) and a wall-jet type ECL detector modified from a commercial Zensor model SF-100 thin-layer electrochemical cell (Taichung, Taiwan). The schematic diagrams of the FI-ECL system and the ECL detector are represented in Fig. 1.

A 1 cm  $\times$  3.5 cm indium/tin oxide (ITO)-coated glass plate (Delta Technologies, Stillwater, MN, USA), situated at the bottom block of the cell, was used as the working electrode for *in situ* generation of the active Ru(bpy)<sub>3</sub><sup>2+</sup>. The potential of the ITO electrode (vs. Ag/AgCl reference) was controlled with a BAS (Bioanalytical System, Inc., West Lafayette, IN, USA) model LC-4C potentiostat. The stainless steel outlet tubing also functioned as a counter electrode. A small cone-shaped hole was drilled on the cell body under the ITO plate, and the emitted ECL was captured using a Hamamatsu R928 photomultiplier tube (PMT; Hamamatsu city, Japan) biased at  $-930$  V and positioned under the detection cell. The photocurrent was amplified by a Keithley model 485 picoammeter (Cleveland, OH, USA), converted to voltage and recorded using a PC equipped with a data acquisition interface. The whole ECL detector was held in a light-tight box constructed from black Plexiglass to exclude stray light.

### 2.2. Chemicals

Glyphosate (GLY, 96%) and tris(2,2'-bipyridyl)ruthenium(II) chloride (Ru(bpy)<sub>3</sub>Cl<sub>2</sub>, 99.95%) were purchased from Aldrich (Milwaukee, WI, USA). All other chemicals were of analytical-reagent grade. All solutions were filtered through a 0.45  $\mu$ m pore-size membrane filter before use.

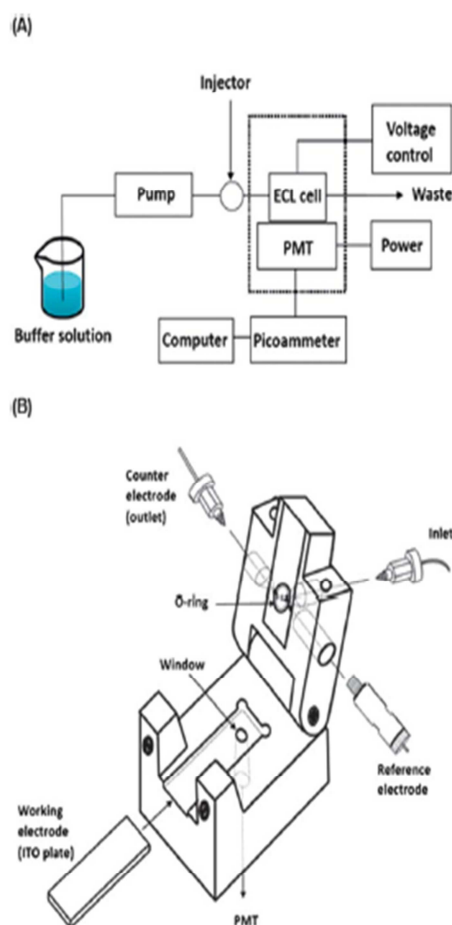


Fig. 1 (A) Schematic diagram of the FI-ECL detection system; (B) exploded view of the ECL detector.

### 2.3. Sample preparation

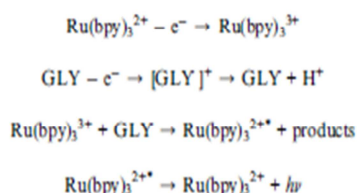
Tap water was acquired from the campus and irrigation water was collected from a local rice field. Waters were collected in clean polypropylene (PP) bottles, acidified to pH 2 with nitric acid, filtered through a 0.45  $\mu$ m pore-size nylon membrane filter and stored at 4  $^{\circ}$ C. Before analysis, 3 mL of water sample was passed through a Varian Bond Elut SCX column (500 mg/3 mL; H<sup>+</sup> form) at a rate of 0.5 mL min<sup>-1</sup>. A 100  $\mu$ L working solution was prepared by mixing 10  $\mu$ L of  $40 \times 10^{-3}$  mol L<sup>-1</sup> aqueous Ru(bpy)<sub>3</sub><sup>2+</sup> solution with 90  $\mu$ L of the above percolate. This solution was used for FI-ECL analysis. The GLY-spiked sample was prepared by adding appropriate amounts of GLY standard solution into the acidified water sample prior to membrane filtration and solid-phase extraction pre-treatment.

## 3. Results and discussion

### 3.1. Characteristics of the FI-ECL system

It is known that the CL emission can be generated *via* reaction between tertiary or some secondary amines and Ru(bpy)<sub>3</sub><sup>2+</sup>. The

latter can be formed by either chemical or electrochemical oxidation of  $\text{Ru}(\text{bpy})_3^{2+}$ .<sup>20</sup> However, the active  $\text{Ru}(\text{bpy})_3^{3+}$  reagent produced by the chemical method often suffers from the problem of limited temporal stability with respect to reduction. Despite that some on-line chemical generation systems of  $\text{Ru}(\text{bpy})_3^{3+}$  had been developed to overcome the problem, to date, electrochemical oxidation is still the most predominant means of  $\text{Ru}(\text{bpy})_3^{3+}$  production which can provide a reliable and constant source of  $\text{Ru}(\text{bpy})_3^{3+}$ . Since GLY is structurally a secondary amine, the possible mechanism of the CL reaction between GLY and electrochemically generated  $\text{Ru}(\text{bpy})_3^{3+}$  can be depicted as follows:<sup>21</sup>



In conventional FI with  $\text{Ru}(\text{bpy})_3^{2+}$ -based CL<sup>22,23</sup> or ECL<sup>24</sup> detection, a manifold flow assembly was always used to continuously deliver the buffer carrier,  $\text{Ru}(\text{bpy})_3^{2+}$  reagent, and catalyst to the reaction/detection zone. In our system, just a single flow channel was employed to deliver the buffer solution while the  $\text{Ru}(\text{bpy})_3^{2+}$  reagent was concomitantly injected with the sample solution via a six-port loop injector. With this flow scheme, significant reduction in the consumption of the expensive  $\text{Ru}(\text{bpy})_3^{2+}$  reagent can be achieved. Besides, flow rate adjustment is much easier.

The wall-jet type ECL detector was modified from a commercial thin-layer electrochemical cell specifically designed for use with disposable screen-printed electrodes (SPEs).<sup>24</sup> The cell was made of foldable polyoxymethylene thick platelets with the bottom portion consisting of a cavity track to drag the SPE in position (see Fig. 1B). Since the chosen ITO-coated glass plate has the same thickness (0.7 mm) as the disposable SPE, if cut to the appropriate length and width, it can readily fit the tray-cavity arrangement of the cell assembly. The wall-jet design through the center micro-hole can guide the inlet eluent to function properly on the ITO working electrode. In order to collect the emitted ECL from the back of the transparent ITO glass plate, a small cone-shaped hole was drilled on the bottom block of the cell assembly precisely opposite to the inlet micro-hole on the top block. The major advantage of this button-and-lock type ECL detector is its ease of assembling the detection cell and changing the sensing electrode.

### 3.2. Optimization of experimental conditions

The observed CL intensity in the FI-ECL system was dependent on the operating parameters such as concentration of  $\text{Ru}(\text{bpy})_3^{2+}$ , detection potential, pH, and flow rate of buffer carrier. During optimization, effects of various parameters were investigated using a univariate approach. All the studies were performed with a 2.1 mg L<sup>-1</sup> GLY standard solution.

**Table 1** Optimal conditions for FI-ECL analysis of GLY

Parameter	Condition
Carrier solution	20 × 10 <sup>-3</sup> mol L <sup>-1</sup> sodium phosphate buffer
pH of buffer solution	9.0
$\text{Ru}(\text{bpy})_3^{2+}$ concentration	4.0 × 10 <sup>-3</sup> mol L <sup>-1</sup>
Flow rate	0.26 mL min <sup>-1</sup>
ITO electrode potential	1.6 V (vs. Ag/AgCl reference)
Voltage of PMT	-930 V

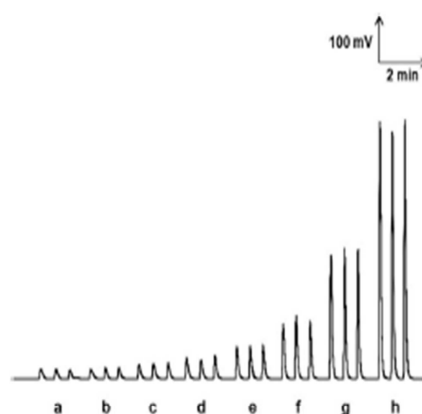
The effect of  $\text{Ru}(\text{bpy})_3^{2+}$  concentrations on the ECL intensity of GLY was first examined in the concentration range of 0.2–6.0 × 10<sup>-3</sup> mol L<sup>-1</sup>  $\text{Ru}(\text{bpy})_3^{2+}$ . The results showed that the ECL intensity increased continuously with increasing  $\text{Ru}(\text{bpy})_3^{2+}$  concentration. A relatively constant peak size was observed after the  $\text{Ru}(\text{bpy})_3^{2+}$  concentration reached 4.0 × 10<sup>-3</sup> mol L<sup>-1</sup>. An injected sample solution of GLY concomitantly containing 4.0 × 10<sup>-3</sup> mol L<sup>-1</sup>  $\text{Ru}(\text{bpy})_3^{2+}$  was chosen as the optimal sample.

The optimal detection potential for GLY was investigated in the range of 1.0–2.0 V (vs. Ag/AgCl reference). The results indicated that the largest ECL signal was obtained at 1.6 V. This potential was therefore selected as the optimal potential.

It is known that the ECL intensity for the reaction of  $\text{Ru}(\text{bpy})_3^{2+}$  with amines is dependent upon pH values. The effect of buffer pH on the ECL of GLY was examined in the pH range of 7–10. With a 20 × 10<sup>-3</sup> mol L<sup>-1</sup> sodium phosphate buffer as the carrier solution, the largest signal was obtained at pH 9.0. Accordingly, this pH was selected as the optimal.

The effect of flow rate on the ECL intensity of GLY was investigated in the range of 0.10–0.56 mL min<sup>-1</sup>. It was found that the ECL intensity of GLY increased with increasing flow rate in the range of 0.10–0.26 mL min<sup>-1</sup>. When the rate was higher than 0.26 mL min<sup>-1</sup>, the ECL intensity decreased, probably due to the less contact time between the analyte and the ITO electrode surface under a high flow rate. Therefore, a flow rate of 0.26 mL min<sup>-1</sup> was used for all analyses.

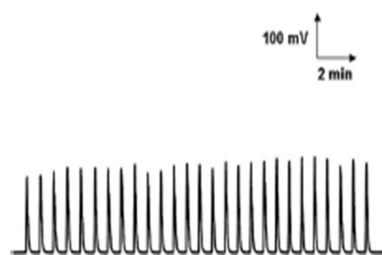
The optimal conditions for FI-ECL analysis of GLY are listed in Table 1.



**Fig. 2** FI-ECL responses of GLY. Concentrations of GLY (mg L<sup>-1</sup>): (a) 0; (b) 0.03; (c) 0.06; (d) 0.14; (e) 0.28; (f) 0.56; (g) 1.40; (h) 2.80.

**Table 2** Comparison of flow-injection methods for the analysis of GLY

Detection	Linear range (mg L <sup>-1</sup> )	LOD (mg L <sup>-1</sup> )	Sample	Reference
Chemiluminescence	0.002–0.034	0.001	Industrial/commercial formulations	25
Fluorescence	0.043–4.3	0.014	Soil and sediment	26
Spectrophotometry	0.5–10	0.17	Water	27
Electrochemiluminescence	0.03–2.81	0.03	Water	This work

**Fig. 3** Repeatability of continuous injection of 1.0 mg L<sup>-1</sup> GLY ( $n = 27$ ).

### 3.3. Analytical performance

Under the optimum conditions, a calibration graph was constructed in the concentration range of 0.03–2.81 mg L<sup>-1</sup> GLY. After subtracting the average peak height of the blank, the corrected peak heights (mV) were plotted against the concentration (mg L<sup>-1</sup>) of GLY analyzed. Using the least-square method, the regression equation was found to be  $y = 189.3x - 1.3$  ( $R^2 = 0.9998$ ;  $n = 7$ ). Fig. 2 shows typical FI-ECL responses of GLY standards. The limit of detection (LOD), based on  $3 \times$  standard deviation of blank height, was calculated to be 0.03 mg L<sup>-1</sup> GLY. This detection sensitivity is similar to that obtained with the HPLC-ECL method,<sup>28</sup> but somewhat inferior to that obtained with the FI-CL method using on-line chemically generated Ru(bpy)<sub>3</sub><sup>3+</sup>.<sup>25</sup> Comparison of linear range and LOD values for GLY among various FI methods is shown in Table 2. Further, a lower LOD value with the present method can be obtained by increasing the volume of sample solution injected; however, the present LOD has been adequate enough for screening residual GLY in drinking water and surface water at the MAC levels set by various countries.<sup>4,7</sup> Repeatability of the method was examined by 27 consecutive injections of 1.0 mg L<sup>-1</sup> GLY into the FI-ECL system, as illustrated in Fig. 3. The RSD of peak height was calculated to be 5.3%. The sample throughput was about 100 injections h<sup>-1</sup>.

### 3.4. Interferences

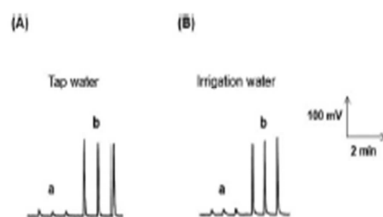
The possible interference of foreign ions in water was studied by analyzing a standard solution of 1.0 mg L<sup>-1</sup> GLY to which various amounts of salts containing foreign ions had been added. The obtained signals were compared with a pure GLY standard at the same concentration. A species was considered not to interfere if it caused a relative signal change less than 5%. The effect of possible interference on GLY analysis is shown in Table 3.

It can be seen that the most commonly found cations and anions in natural waters did not interfere at a 200-fold concentration ratio of foreign ions to GLY. Glufosinate, another commonly used phosphonic and amino acid group-containing pesticide, and aminomethylphosphonic acid (AMPA), the major metabolite of GLY, also did not interfere. Since natural organic matter (NOM) concentration in surface water typically ranges from 0.1 mg L<sup>-1</sup> to 20 mg L<sup>-1</sup> and is mainly composed of humic substances,<sup>25</sup> humic acids at 10 mg L<sup>-1</sup> level were examined to assess the possible effect of NOM on the analysis of GLY in water. No interference due to the co-existence of humic acids was observed. However, a significant suppression of ECL signal intensity due to Fe<sup>2+</sup>, Ca<sup>2+</sup>, Pb<sup>2+</sup>, Cu<sup>2+</sup>, Al<sup>3+</sup> and Fe<sup>3+</sup> was found, probably due to the formation of complexes between phosphate-bearing GLY and some multivalent cations.<sup>26</sup> Most interferences caused by divalent cations can be effectively eliminated by first acidifying the water sample to pH 2 followed by pretreating the sample with a disposable solid-phase extraction column packed with strong cation-exchange (SCX) materials. Results indicated that interference in the detection of 1.0 mg L<sup>-1</sup> GLY standard was insignificant (<5%) at a 200-fold concentration ratio of interfering cations to GLY if the acidified water sample was passed through a Bond Elut SCX column (Varian) in the H<sup>+</sup> form prior to FI-ECL analysis. Interference due to trivalent cations, e.g. Al<sup>3+</sup> and Fe<sup>3+</sup>, was more troublesome. We found that the tolerance concentration ratios for Al<sup>3+</sup>/GLY and Fe<sup>3+</sup>/GLY at 1.0 mg L<sup>-1</sup> GLY level were 50 and 20, respectively. Generally, in waters with higher dissolved organic carbon (DOC), such as surface water, some of these

**Table 3** Effect of coexisting ions on ECL intensity of GLY at 1.0 mg L<sup>-1</sup>

Species	Concentration (mg L <sup>-1</sup> )	Change in ECL intensity
Na <sup>+</sup> , K <sup>+</sup> , Mg <sup>2+</sup> , Ba <sup>2+</sup> , Zn <sup>2+</sup> , Cd <sup>2+</sup> , Cl <sup>-</sup> , NO <sub>3</sub> <sup>-</sup> , HCO <sub>3</sub> <sup>-</sup> , HSO <sub>4</sub> <sup>-</sup> , H <sub>2</sub> PO <sub>4</sub> <sup>-</sup> , CO <sub>3</sub> <sup>2-</sup> , SO <sub>4</sub> <sup>2-</sup> , glufosinate, AMPA <sup>a</sup>	200	<5%
Fe <sup>2+</sup>	200	-9%
Ca <sup>2+</sup>	200	-18%
Pb <sup>2+</sup>	200	-35%
Cu <sup>2+</sup>	200	-52%
Al <sup>3+</sup>	200	-49%
	50	<5%
Fe <sup>3+</sup>	200	-76%
	20	<5%
Humic acids	10	<5%

<sup>a</sup> AMPA = aminomethylphosphonic acid.



**Fig. 4** Analysis of GLY in (A) tap water and (B) rice field irrigation water. Signal identities: (a) original water; (b) water spiked with 1.0 mg L<sup>-1</sup> GLY.

interferences will be reduced due to a partial complexation of interfering cations in the organic matter.

### 3.5. Applications

The applicability of the method was examined by analyzing GLY in two authentic samples, viz., tap water and rice-field irrigation water. The results are shown in Fig. 4. No detectable GLY was found in either water sample. On the other hand, a significant ECL signal could be observed in waters fortified with 1.0 mg L<sup>-1</sup> GLY. Recoveries of spiked GLY were 97% and 95% for tap water and irrigation water, respectively, as determined with the method of standard addition.

## 4. Conclusion

A flow-injection method coupled with Ru(bpy)<sub>3</sub><sup>2+</sup>-ECL detection for analyzing trace GLY in water has been developed. The single flow channel FI system including the wall-jet type ECL detector was easily assembled. As a screening method for residual GLY in water, this method has the advantages of simple apparatus, selective and sensitive detection capability, and high sample throughput. Besides, screening water samples for GLY at the MAC level can be readily performed without any derivatization or pre-concentration treatment. In comparison with the similar FI-CL method using the chemically generated active reagent Ru(bpy)<sub>3</sub><sup>2+</sup>, the main advantages of this FI-ECL method include significant reduction in consumption of the expensive Ru(bpy)<sub>3</sub><sup>2+</sup> reagent and simple flow assembly. This method is applicable to the rapid screening of residual GLY in drinking water and surface water.

## Acknowledgements

Financial support from the National Science Council of Taiwan is gratefully acknowledged.

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# 科技部補助計畫衍生研發成果推廣資料表

日期:2014/08/13

科技部補助計畫	計畫名稱: 磁固相萃取方法的發展
	計畫主持人: 黃承文
	計畫編號: 100-2113-M-029-002-MY3      學門領域: 分離科學
無研發成果推廣資料	

100 年度專題研究計畫研究成果彙整表

計畫主持人：黃承文		計畫編號：100-2113-M-029-002-MY3					
計畫名稱：磁固相萃取方法的發展							
成果項目		量化			單位	備註（質化說明：如數個計畫共同成果、成果列為該期刊之封面故事...等）	
		實際已達成數（被接受或已發表）	預期總達成數（含實際已達成數）	本計畫實際貢獻百分比			
國內	論文著作	期刊論文	0	0	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%		
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（本國籍）	碩士生	0	0	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	0	0	100%		
國外	論文著作	期刊論文	3	3	100%	篇	
		研究報告/技術報告	0	0	100%		
		研討會論文	0	0	100%		
		專書	0	0	100%	章/本	
	專利	申請中件數	0	0	100%	件	
		已獲得件數	0	0	100%		
	技術移轉	件數	0	0	100%	件	
		權利金	0	0	100%	千元	
	參與計畫人力（外國籍）	碩士生	2	2	100%	人次	
		博士生	0	0	100%		
		博士後研究員	0	0	100%		
		專任助理	1	1	100%		

<p>其他成果 (無法以量化表達之成果如辦理學術活動、獲得獎項、重要國際合作、研究成果國際影響力及其他協助產業技術發展之具體效益事項等，請以文字敘述填列。)</p>	<p>無</p>
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	成果項目	量化	名稱或內容性質簡述
科 教 處 計 畫 加 填 項 目	測驗工具(含質性與量性)	0	
	課程/模組	0	
	電腦及網路系統或工具	0	
	教材	0	
	舉辦之活動/競賽	0	
	研討會/工作坊	0	
	電子報、網站	0	
	計畫成果推廣之參與(閱聽)人數	0	

# 科技部補助專題研究計畫成果報告自評表

請就研究內容與原計畫相符程度、達成預期目標情況、研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）、是否適合在學術期刊發表或申請專利、主要發現或其他有關價值等，作一綜合評估。

1. 請就研究內容與原計畫相符程度、達成預期目標情況作一綜合評估

達成目標

未達成目標（請說明，以 100 字為限）

實驗失敗

因故實驗中斷

其他原因

說明：

2. 研究成果在學術期刊發表或申請專利等情形：

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其他：（以 100 字為限）

3. 請依學術成就、技術創新、社會影響等方面，評估研究成果之學術或應用價值（簡要敘述成果所代表之意義、價值、影響或進一步發展之可能性）（以 500 字為限）

本計畫發展出以 Fe<sub>3</sub>O<sub>4</sub>@Al<sub>2</sub>O<sub>3</sub> 奈米粒子為基礎的磁固相萃取方法，適合用來萃取含磷酸基團的有機化合物，例如：單磷酸基的 glyphosate；雙磷酸基的 alendronate 與 ibandronate。配合毛細管電泳分離及光學偵測技術，本計畫成功地完成以下 3 項工作：(1) 以毛細管電泳/電激發化學發光法配合 Fe<sub>3</sub>O<sub>4</sub>@Al<sub>2</sub>O<sub>3</sub> 磁奈米粒子固相萃取分析藥物及尿液樣品中的 ibandronate；(2) 以毛細管電泳/螢光偵測法配合 Fe<sub>3</sub>O<sub>4</sub>@Al<sub>2</sub>O<sub>3</sub> 磁奈米粒子固相萃取分析尿液與血漿樣品中的 alendronate；(3) 以流動注入法配合電激發化學發光快速篩檢水中 glyphosate 除草劑。三項研究成果皆已發表於分析化學領域國際期刊。本計畫成果兼具學術與應用價值。