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Full Paper

Pulsed Amperometry for Anti-fouling of Boron-doped Diamond in Electroanalysis of β-Agonists: Application to Flow Injection for Pharmaceutical Analysis

Chanpen Karuwan [1,2], Thitirat Mantim [1,2], Patcharin Chaisuwan [1,2], Prapin Wilairat [1,2], Kate Grudpan [3], Piyada Jittangprasert [1,4], Yasuaki Einaga [5], Orawon Chailapakul [6], Leena Suntornsuk [7], Oraphan Anurukvorakun [7] and Duangjai Nacapricha [1,2,*]

[1] Flow Innovation-Research for Science and Technology Laboratories (FIRST Labs.), Faculty of Science, Mahidol University, Rama 6 Road, Bangkok, 10400 Thailand
[2] Department of Chemistry, Faculty of Science, Mahidol University, Bangkok, Thailand
[3] Department of Chemistry, Faculty of Science, Chiang Mai University, Chiang Mai, Thailand
[4] Department of Chemistry, Faculty of Science, Srinakharinwirot University, Bangkok, Thailand
[5] Department of Chemistry, Faculty of Science and Technology, Keio University, Japan
[6] Department of Chemistry, Faculty of Science, Chulalongkorn University, Bangkok, Thailand
[7] Department of Pharmaceutical Chemistry, Faculty of Pharmacy, Mahidol University, Thailand
* Author to whom correspondence should be addressed; E-mail: scdnc@mahidol.ac.th and dnacapricha@gmail.com

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Abstract: This work presents the construction and application of boron-doped diamond (BDD) thin film electrode as sensor for the determination of three β-agonists, viz. salbutamol, terbutaline and clenbuterol. Although well-known as a chemically inert material, BDD film however shows fouling in detection of these compounds using fixed-potential mode amperometry. A suitable waveform for pulsed amperometric detection (PAD) was developed and used to determine the agonist compounds. It was seen that the developed PAD significantly refreshed the BDD surface for long-term detection in flow injection analysis. Linear working ranges were 0.5-100 μM, 1.0-100 μM and 0.5-50 μM for salbutamol, terbutaline and clenbuterol, respectively. The developed PAD-BDD system was applied to successfully determine salbutamol and terbutaline in commercial pharmaceutical products. The methods were validated with a capillary electrophoresis method.

Keywords: boron-doped diamond, pulsed amperometry, β-agonists, flow injection

1. Introduction

Diamond thin-film electrodes have been used as sensors in various applications of electroanalysis [1-14]. Synthesis of the polycrystalline film of diamond is carried out on the surface of a substrate in a close system. The substrates can be quartz, molybdenum, tungsten, platinum or silicon. Based upon two major considerations (temperature tolerance and similar thermal expansion coefficient to diamond), silicon wafer is often employed [2]. To make the diamond electrically conductive, specific impurities are added during formation of the thin-film on the substrate. The common dopant is boron and this type of electrode is called boron-doped diamond (BDD). BDD films can be produced synthetically by low pressure methods with the most popular method being chemical-vapor deposition (CVD) [1,2].

Thin film BDD on Si provides benefits over sp^2-type carbon electrodes, e.g., carbon paste and glassy carbon (GC) [1-2]. Excellent properties of the BDD for most electrolytes include (i) low and stable background current; (ii) significantly wide working potential window; (iii) considerably low capacitance; (iv) low adsorption of polar molecules (unlike GC electrode) [7]; (v) excellent reproducibility [8]. In view of these impressive properties, BDD have been employed as the sensor in flow injection (FI) analysis [9-13] and in liquid chromatography [11,14]. All these were carried out using amperometric detection at the BDD electrode.

Although there have been quite a number of publications concerning the use of BDD in flow-based detection, there has been no published work on beta-adrenergic agonists. Beta-adrenergic agonists, or β-agonists, are used in human in the treatment of asthama. β-agoinists are also illegally used as animal feed additives. This is because β-agonists increase protein accretion and decrease lipogenesis [15]. β-agonists can also produce anabolic like effect and therefore its use is classified by the World Anti-Doping Agency (WADA) as '*prohibited drugs in sports'* [16].

Due to the necessity to detect and monitor β-agonists, several methods have been developed for determination of these compounds, including gas chromatography with mass spectrometric detection (GC-MS) [17,18] and high performance liquid chromatographic (HPLC) method with UV [19], electrochemical [20] and MS [21,22] detections. Some reported methods are based on capillary electrophoresis (CE) with MS [23] and amperometry [24] detection.

Amongst all techniques of detection, electrochemical detection has shown to be very selective, sensitive and yet simple. The technique is capable of detecting non-chromophoric or non-fluorophoric compounds. Amperometric detection has been employed by some authors for analysis of β-agonists [20, 24-26]. The most popular working electrode is the carbon electrode, including glassy carbon [20, 25], carbon disk [24] and carbon paste [26] electrodes. Like other analytes, there are some reports of electrode fouling in the oxidation detection of β-agonists at carbon surface [20, 25]. To overcome this fouling problem, cleaning of electrode surface is carried out by mechanical polishing or electropolishing. Electropolishing is more convenient because it can be employed at any time during analysis.

Pulsed amperometric detection (PAD) is a type of electropolishing, which utilizes a potential waveform to generate new electroactive electrode surface prior to each current measurement. Unlike common amperometric measurement, the applied potential waveform is an alternating potential

between positive and negative values [27]. This cleans and reactivates the electrode surface. The frequency of potential pulse is approximately 1 Hz.

In this work, we used an in-house BDD electrode for amperometric detection and quantitation of three β-agonists, viz. salbutamol, terbutaline and clenbuterol (Fig 1.).

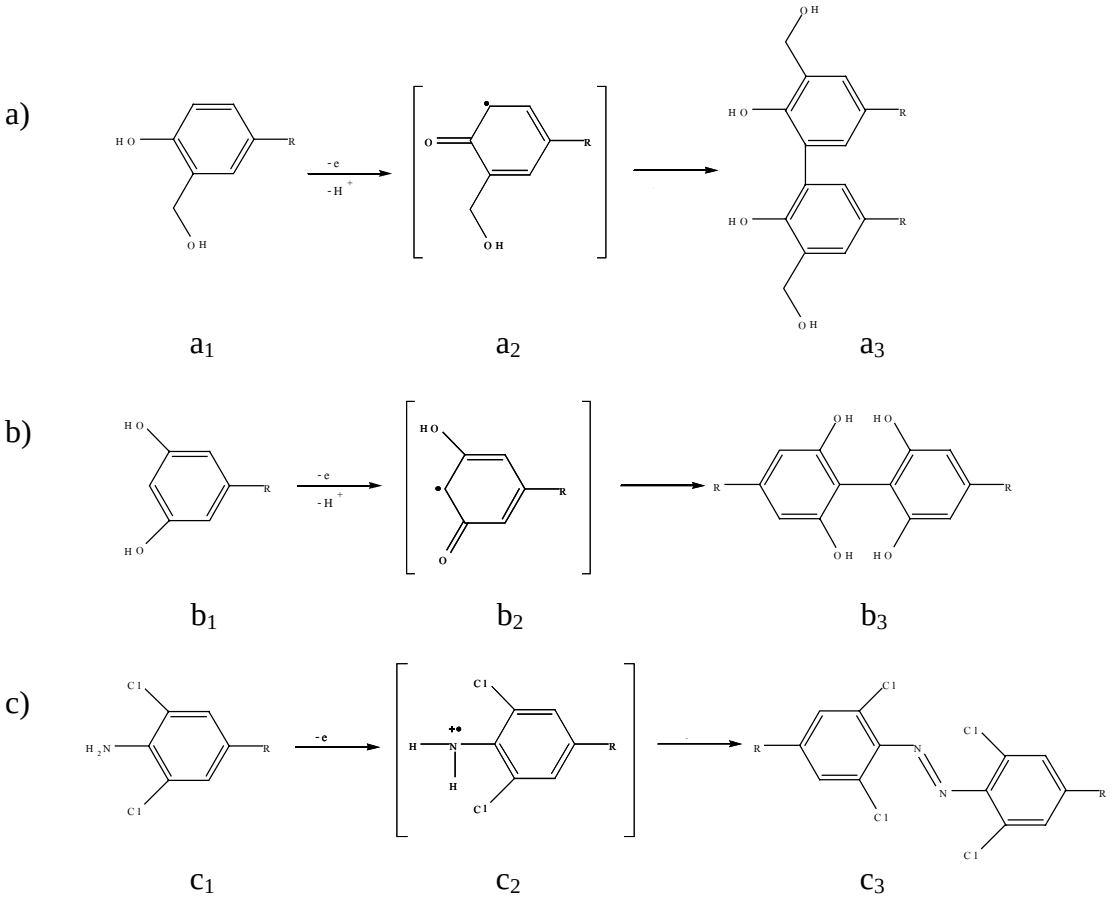


Figure 1. The structures and oxidation reactions of the three β-agonists: (a) Salbutamol (b) Terbutaline (c) Clenbuterol, and their reduced (a_1, b_1 and c_1), intermediate (a_2, b_2 and c_2), and oxidized (a_3, b_3 and c_3) forms [28-30].

Although BDD often provides outstanding properties, compared to other carbon electrodes, we observed that all oxidative products of β-agonists fouled the BDD electrode when used in a fixed-potential amperometry mode. Amperometric detection even with continuous-flow did not completely eliminate the adsorption. PAD was then investigated in order to solve the problem. The final optimized PAD waveform was then applied with flow injection for determination of the β-agonists in drug formulations.

2. Experimental

2.1 The BDD Electrode

The BDD films were grown on Si(100) substrates by microwave-assisted plasma CVD technique using a commercial microwave plasma reactor (ASTex Corp., Woburn, MA) at 5 kW. The carrier gas was high purity hydrogen. B_2O_3 was used as the source for boron. B_2O_3 was dissolved in a mixture of acetone-methanol mixture (ratio of 9:1, v/v). This gives B/C molar ratio of approximately 1:100. The film thickness was approximately 40 μm after 10 h of CVD deposition. Following the usual procedure, the quality of the film was confirmed by Raman spectroscopy. Details of the preparation have been described elsewhere [8].

Prior to using the BDD, the electrode was sonicated in 2-propanol for 5 min, followed by rinsing with deionized-distilled water.

2.2 Cyclic voltammetry

A potentiostat, μ-Autolab Type III (Metrohm, Switzerland), with single compartment three electrode glass cell, was used for all the cyclic voltammetric (CV) studies. A BDD electrode was pressed against the smooth ground joint at the base of the cell and isolated by an o-ring, producing 0.07 cm^2 electrode surface. Electrical contact was made by placing the underside of the electrode (Si substrate) on a brass plate. The electrochemical cell was housed in a faraday cage to reduce electrical noise. A platinum wire and Ag/AgCl reference electrode (3 M KCl) were used as the auxiliary and reference electrodes, respectively.

The CV behaviour of three β-agonists was studied using BDD and glassy carbon electrodes. A scan rate of 100 mV s^{-1} was employed for all CV scans. The scans were made from 0.00 to 1.00 V.

2.3 Amperometric detection by flow injection

In the development of analysis procedure using BDD as sensor for β-agonists, the flow injection manifold shown in Fig. 2 was utilized. An Ismatec peristaltic pump (model IS7610, Switzerland) was used for propelling the carrier stream (buffer). Arrangement of the amperometric detector with BDD as working electrode was described in previous works [9-14].

Initially, amperometric detection of agonist compounds was carried out for PAD using the manifold in Fig. 2, operated at a constant potential. The same system was employed using a pulsed potential program, controlled by the potentionstat. The optimization studies as well as the optimal conditions, for the pulse mode, are shown in Table 1.

2.4 Chemicals

All chemicals used in this work were of analytical reagent grade. Deionized-distilled water was used throughout. Tris buffer solution (pH 8.3) was prepared from tris (hexahydroxy) aminomethane (Merck, Germany) and 0.1 M HCl (Lab Scan, Ireland). Standard solutions of salbutamol, terbutaline and clenbuterol (all from Sigma, USA) were prepared in 50 mM tris buffer solution.

2.5 Sample preparation

Four pharmaceutical products containing salbutamol and terbutaline were purchased from local drug stores. These drugs are for treatment of asthma. 'Sabumol' and 'Bricanyl' were in syrup form. These samples were diluted in 50 mM tris buffer pH 8.3 before direct analysis (1/40 dilution for 'Sabumol' and 1/20 for 'Bricanyl'). 'Ventolin' and 'Fasma' tablets were ground before extraction with water, followed by centrifugation and filtration through 0.45 micron cellulose acetate membrane. The filtrates were suitably diluted with 50 mM tris buffer pH 8.3.

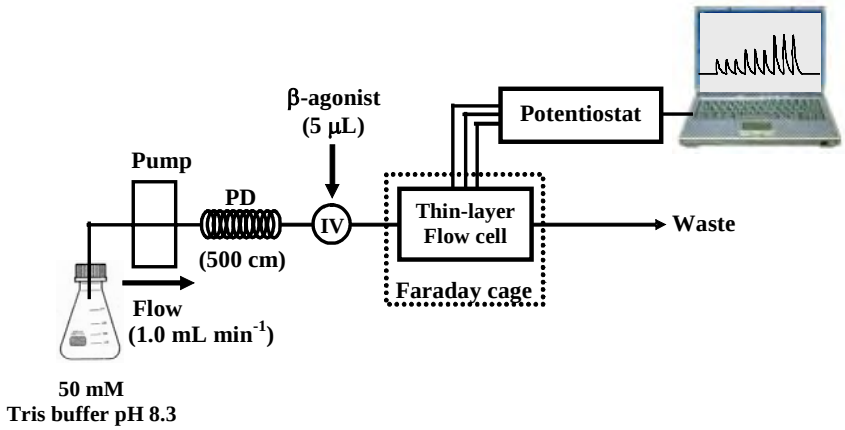


Figure 2. The flow injection manifold used in all amperometric measurements, including pulsed amperometry. PD: pulse dampener. IV: injection valve. The condition depicted is optimal configuration with Ag/AgCl and Pt as the reference and the auxiliary electrodes, respectively.

2.6 Analysis of samples by capillary electrophoresis

A capillary electrophoresis (CE) method, developed for quantitative analysis of the β-agonists (to be described elsewhere), was adopted for method validation. The CE instrument (Agilent Technologies, USA) was operated in a non-aqueous system. The capillary was a fused-silica (50 μm, i.d.), with total length of 64.5 cm (effective length, 56 cm), thermostatted at 24^{o}C. UV detection wavelength was 220 nm. The background electrolyte was 18 mM CH_3COONH_4 in methanol; acetonitrile; glacial acetic acid (66:33:1, v/v). Injection time, with siphoning action, was 4 seconds. Applied voltage was 28 kV.

Standard stock solutions of salbutamol and terbutaline (approx. 100 ppm) were accurately prepared in methanol. Chlorpheniramine maleate (CPM) was used as the internal standard. Calibrations were constructed in the range of 1-20 ppm, using peak area ratios.

3. Results and Discussion

3.1 Cyclic voltammetry

3.1.1 Performance of BDD compared to GC

As in previous reports [9-12, 31-32], the background voltammogram of BDD, using our buffer system, was much better than the background of GC electrode (Fig. 3a). This low background current of BDD, shown in Fig. 3a, provides a more sensitive system than the conventional GC system for analysis of β-agonists.

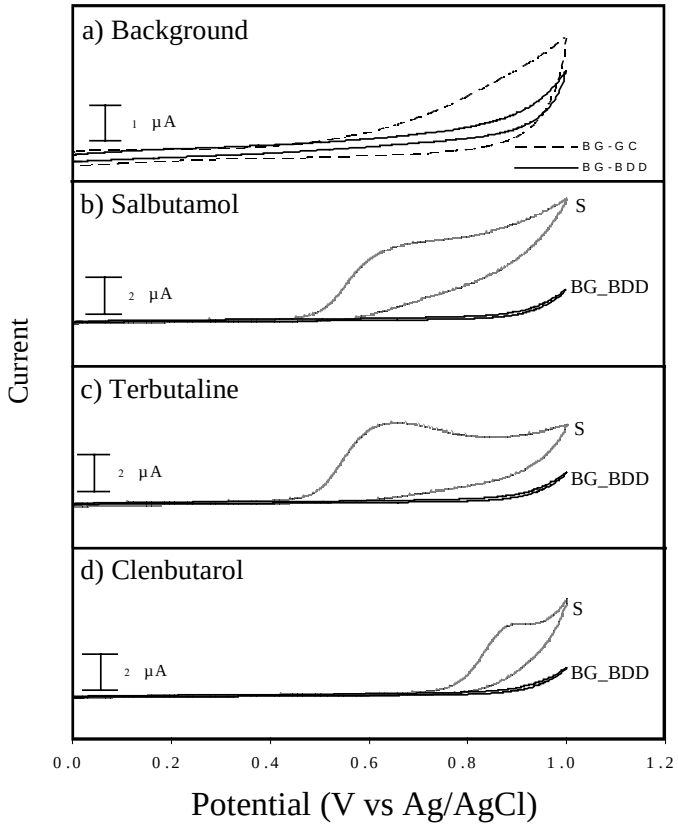


Figure 3. Background (BG) and signal (S) voltammograms obtained from 1 mM standard solutions of salbutamol, terbutaline and clenbuterol. Working electrode: BDD or GC. Auxiliary electrode: Pt. Buffer: 50 mM Tris buffer pH 8.3. Scan rate was 100 mV/s.

3.1.2 Selection of buffer

Four buffers were selected from the literature, for capillary electrophoresis of the β-agonists [15, 33-34]. The buffers tested (all 50 mM) were (i) phosphate buffer at pH 2.4 [33], (ii) phosphate buffer at pH 6.6 [34], (iii) Tris buffer at pH 5.0 [15] and (iv) Tris buffer at pH 8.3 [15]. Cyclic voltammetric studies were carried out for salbutamol, from 0-1.2 volts (results are not shown). For the first three buffers, no well-defined peak currents were obtained. Only the Tris buffer pH 8.3 gave a well-defined, cyclic voltammogram. Thus this buffer was selected for further studies.

Cyclic voltammograms of salbutamol, terbutaline and clenbuterol standard solutions (in Tris buffer pH 8.3) are shown in Fig. 3b, 3c and 3d, respectively. The voltammograms, for all the analytes, gave well-defined oxidation peaks, but irreversible. In the selected buffer, oxidation peak potentials (vs Ag/AgCl) were observed at 0.6 V (for salbutamol and terbutaline) and at 0.9 V (for clenbuterol).

3.2 Amperometric flow injection experiments with BDD

3.2.1 Fouling of BDD

The oxidative peak potentials were used with the FI manifold (Fig. 2), for preliminary experiment of hydrodynamic voltammetry. Replicate injections were made for all the standard solutions of agonists. It was observed that the oxidative products resulted in serious BDD fouling. An example of the fouling from injections of salbutamol standards is displayed in Fig. 4a. The precision obtained from the results of salbutamol was very poor.

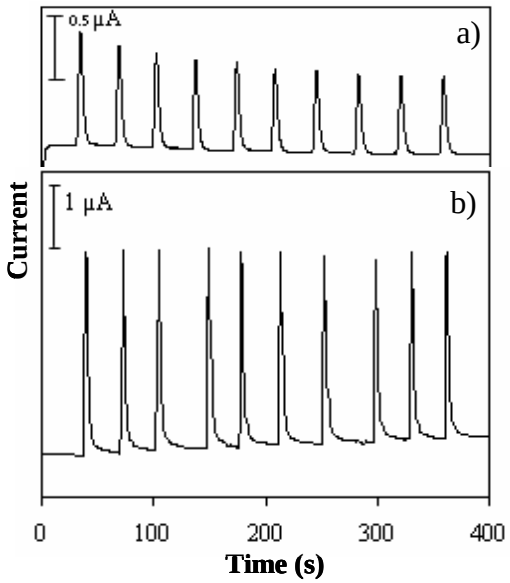


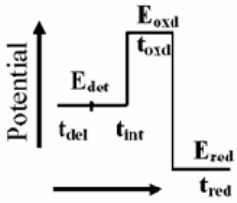
Figure 4. Examples of fouling (a) and anti-fouling (b) of BDD electrode for FI detection. Results were obtained from replicate injections of standard salbutamol $1x10^{-5}$ M to the FI manifold in Fig. 2. (a) amperometric FI with detection potential fixed at 0.6 V. (b) pulsed amperometric FI with the optimized condition shown in Table 1.

The fouling could be due to surface adsorption of the oxidized products of the β-agonists. Possible oxidation products are shown in Fig. 1. The product of oxidation of salbutamol has been proposed based from the pathway for phenol [28] and from cited oxidation of the phenolic hydroxyl group of salbutamol [29]. Clenbuterol may be oxidized to the dimer c_3, as formerly reported by Smith G. F. [30]. For terbutaline, it is thought that direct coupling take place to form product b_3.

Some authors suggested that, for glassy carbon electrode in a phosphate buffer, fouling could be worse in acidic medium [25]. Alkaline medium should then improve the precision. However, our electrolyte with the BDD electrode was already alkaline. BDD is well-known as a chemically inert

surface. Therefore, a protocol for eliminating the fouling from adsorption phenomena (Fig. 4a), should be investigated.

Table 1. Summary of the waveform for optimization PAD-BDD and the optimal condition for salbutamol (s), terbutaline (t), clenbuterol (c).

Wave form		Potential (V)			Time (s)	
		Studied range	Optimum		Studied range	Optimum
	E_{det}	0.5 – 1.0	$0.6^{s,t}$, 0.9^{c}	t_{del}	0.03 – 0.6	0.03
				t_{int}	0.03 – 0.2	0.03
	E_{oxd}	1.0 – 1.4	1.3	t_{oxd}	0.03 – 1.0	1.0
	E_{red}	-0.2 – 0.3	– 0.1	t_{red}	0.03 – 0.8	0.08

E_{det} : detection potential
E_{oxd} : oxidation potential
E_{red} : reduction potential
t_{del} : delay time
t_{int} : integration time
t_{oxd} : oxidation time
t_{red} : reduction time

3.2.2 Electropolishing by PAD and waveform optimization

Previous works have shown that pulsed potential cleaning, as carried out in PAD technique, is an effective method for electropolishing in analysis of tetracycline [35], doxycycline and chlortetracycline [36]. Some authors have reported use of the PAD technique for BDD and GC electrodes [35, 37]. We thus implemented the technique of PAD in this work for cleaning the surface of electrode.

The PAD waveform used in this work is described in Table 1. Generally, E_{det} is the detection potential applied for the time period t_{det} (t_{det} = t_{del} + t_{int}). The electrode current is detected and integrated over the time period t_{int}, following a delay time t_{del}, to allow the charging current to decrease to an insignificant value. A positive cleaning potential (E_{oxd}) that removes the oxidizable contaminant on the electrode surface, is applied for the time period t_{oxd}. A negative reactivating potential (E_{red}) is applied for the time period t_{red}, following the E_{oxd}. The optimization of each waveform parameter was carried out by single-parameter variation, using the FI set up in Fig. 2. The studied ranges (E and t) are summarized as shown in Table 1, together with the final selected optimum values.

At the various condition of the PAD waveform (Table 1), there were notable improvements, compared to the condition of constant potential. Introduction of PAD resulted a large improvement, in terms of better precision (Fig. 4b and Fig. 5a) and higher sensitivity (Fig. 4b, Fig. 5b). Thus, detections of β-agonists were carried out using the PAD technique in the following experiments.

3.2.3 Optimization of injection volume

Various injection volumes (5, 20 and 50 μL) of the FI system (Fig. 2) were studied for construction of calibration curves of salbutamol, terbutaline and clenbuterol. We found that the sensitivities (slopes of calibrations) were not significantly different (i.e. independent of the injected volume). However, larger volumes (20 and 50 μL) exhibited poorer correlation coefficients (r^2), 0.90-0.95. This may be due to some surface adsorption, similar to that observed in Fig. 4a. The smallest volume, 5 μL, gave good r^2 (0.99). Therefore, in order to reduce the volume of injection, and more importantly to minimize electrode fouling, an injection volume of 5 μL was selected.

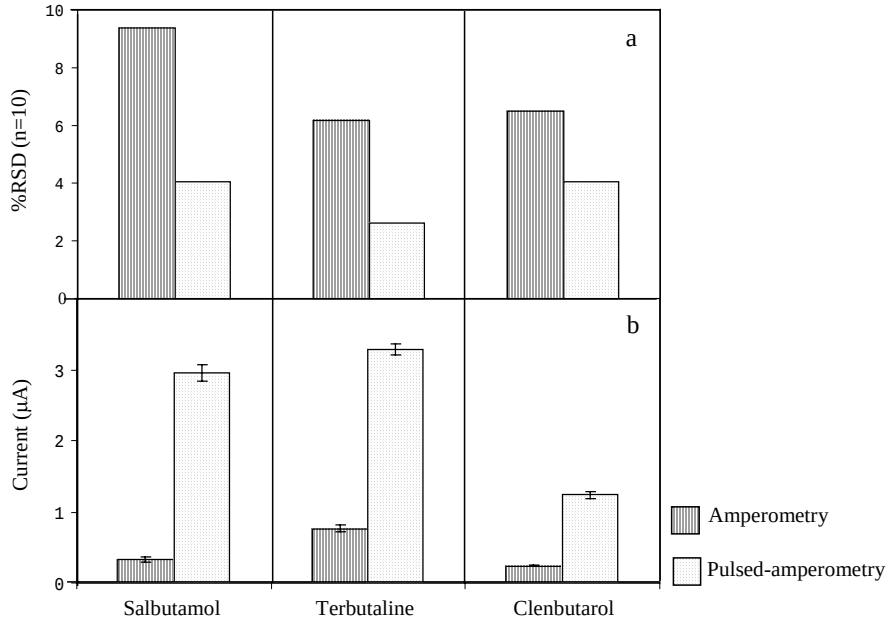


Figure 5. Significant improvement in both precision (a) and signal size (b) upon employment of pulsed amperometry with BDD electrode. Results were from replicate injections of salbutamol, terbutaline and clenbutarol standards ($1x10^2$ μM), using the FI system in Fig. 2.

3.3 Analytical features

We examined various features of our FI-BDD sensing system for analysis of salbutamol, terbutaline and clenbuterol, using the PAD conditions shown in Table 1. Table 2 is a summary of the performance of the BDD sensor for pulsed-amperometric detection of the three β-agonists.

Table 2. Analytical features of the developed flow injection method, with PAD on boron-doped diamond for analysis of β-agonists.

Parameters	Salbutamol	Terbutaline	Clenbuterol
1. Linear range (μM)	0.5-100	1.0-100	0.5-50
2. Regression equation (slope x 10^{-3})	y = (6.2±0.3)x + (15.4±11.8)	y = (6.4±0.4)x + (20.2±23.0)	y = (6.0±0.3)x + (59.0±5.3)
3. Correlation coefficient (r^2)	0.999	0.999	0.999
4. Detection limit (μM: 3S/N)	0.1	0.5	0.3
5. Repeatability (%RSD, n=10)	4.1	2.6	4.0

3.4 Performance for pharmaceutical applications

We tested our BDD sensor for determination of 4 asthma drugs. These drugs are commercially available in Thailand. The experiments were carried out using the system in Fig. 2, operated under the optimized PAD condition (Table 1). Unfortunately, there are no commercial drugs that contain clenbuterol and therefore only formulation containing salbutamol and terbutaline were analyzed.

Table 3. Comparison between the determined and the labeled values of β-agonists in asthma drugs.

Trade name	β-agonist	Type	Unit	Content of agonist		
				Label	CE-UV[a]	PAD-BDD[b] (this method)
1. Sabumol	Salbutamol	syrup	mg/L	400	411 ± 19	408 ± 9
2. Ventolin	Salbutamol	tablet	mg/tablet	2	2.03 ± 0.10	1.91 ± 0.03
3. Bricanyl	Terbutaline	syrup	mg/L	300	287 ± 2.9	306 ± 3.3
4. Fasma	Terbutaline	tablet	mg/tablet	2.5	2.55 ± 0.04	2.40 ± 0.07

[a]Capillary electrophoresis with UV detection

[b]Pulsed amperometric detection on boron-doped diamond by flow injection

Results in Table 3 show agreeable agreement between our PAD-BDD results and the results obtained by the CE method (to be described elsewhere). The results determined by both methods are not significantly different, at 95% confidence by paired *t*-test [38]. The values obtained from the analyses (PAD-BDD and CE) are also very close to the labeled values on the packaging. In this work, commercial drugs in the form of tablet or syrup were used. As seen by the results in Table 3, there are no matrices in the commercial drugs that interfered with the detection by the PAD-BDD method. However, when tested our method with other drugs, it was observed that the drugs' excipients

interfered the PAD-BDD and thus coupling with a separation method may be necessary for such samples.

Recoveries were also carried out, by standard addition during sample preparation, to test our method validity. For salbutamol, %recoveries were 80.8 $\pm$ 7.9 and 86.2 $\pm$ 6.0, for sample 1 and sample 2 in Table 3, respectively. The %recoveries for terbutaline drug were slightly better at 93.1 $\pm$ 5.9 and 91.4 $\pm$ 0.7, for sample 3 and sample 4 in Table 3, respectively.

4. Conclusion

This work exploited another application of the BDD thin-film electrode for quantitative analysis of β-agonists such as salbutamol, terbutaline and clenbuterol. The BDD film was grown on Si substrate by the technique of CVD. This BDD sensor is best operated using pulsed amperometic detection. Unlike other compounds, BDD sensor performs very poorly when operating under constant potential for amperometric detection of the agonist drugs. Fouling of the sensor was found even employed in continuous-flow fashion like flow injection. Pulsing potential, with developed PAD waveform, is efficient to maintain the activity of the BDD surface even over long analysis time. PAD will thus be applied for BDD detection of these agonists in capillary electrophoresis.

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