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Determination of IPTG in Recombinant Human Growth Hormone with Ion Chromatography and Pulsed Electrochemical Detection

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Abstract

A novel method for the determination of isopropyl-β-D-thiogalactopyranoside (IPTG), a sulfur-containing compound, a pharmaceutical additive in commercial recombinant human growth hormone (rhGH), has been developed using pulsed electrochemical detection (PED), following ion Chromatographic separation. A Dionex-500 ion chromatograph coupled with an electrochemical detector was employed, equipped with a gold working electrode and an Acclaim 300 analytical C18 column, with a mobile phase consisting of sodium acetate (NaOAc) buffer (pH 5.45, 0.01 mol/L) and acetonitrile (ACN)(90/10, v/v). Upon optimization, IPTG was found to have a limit of detection of 1 ng/mL (0.1 μmol) with 25 uL injection volume. This method was successfully applied to the determination of IPTG in rhGH samples with the characteristics of simplicity, high sensitivity and good repeatability.

Keywords: Ion chromatograph; Pulsed electrochemical detection; Isopropyl β-D-thiogalactopyranoside

Introduction

Human growth hormone (hGH) consists of 191 amino acid residues folded into a four-helix bundle structure with two disulfide bridges, with a wide range of biological functions as protein synthesis, cell proliferation and metabolism [1,2]. Recombinant human growth hormone (rhGH) is a type of GH produced by recombinant DNA technology identical to human growth hormone [3,4].

Isopropyl-β-D-thiogalactopyranoside (IPTG) as a highly stable molecular biology reagent and a molecular mimic of allolactose to trigger the transcription of the lac operon, is commonly used in cloning procedures to induce the expression of cloned genes which are under the control of the lac operon [5,6]. In the thioether group of IPTG (Figure 1), the sulfur atom creates a chemical bond which is non-hydrolysable by the cell, preventing the cell from degrading the inductant. IPTG has also been widely used in the recombinant processing of rhGH [7,8]. However, the toxicity of IPTG [9,10] restricted the usage of this promoter system, measuring its presence and concentration in the final products is of importance.

Several different techniques have been used for the analysis of thiocompounds [11], such as liquid chromatography, Capillary electrophoresis and HPLC-MS-based methods [12-17], which have attracted much attention in evaluating sulfur-containing compounds because of their good separation and detection ability [12,13]. However, for the absence of strong chromophores or luorophores, the determination of thiocompounds with UV [18,19] or luorescence [20,21] methods should be coupled with derivatization.

Over the past decade, ion chromatography (IC), together with pulsed electrochemical detection (PED), has been accepted [21-24] as highly sensitive and selective methods to detect thiols, disulfides. In addition, both reduced and oxidized thiol moieties can be easily detected without the need of derivatization [12]. Thiocompounds can be detected over a wide range of pH conditions and the response is relatively unperturbed by buffer composition. Integrated pulsed amperometric detection (IPAD) employs more stable baselines, eliminates oxide-induced artifacts, and yields lower limits of detection than those of other PED waveforms in this method.

In this paper, IC coupled with IPAD was applied to test IPTG in biopharmaceutical hormone rhGH. Electrochemical characterization via cyclic voltammetry was performed to optimize the IPAD waveform, and developed to separate IPTG in rhGH samples and then determine it with PED.

Experimental

Chemicals, reagents, standard solution and sample preparation

All solutions were prepared from reagent grade chemicals. HPLC grade sodium acetate (NaOAc) was from Fluka (Steinheim, Germany). HPLC-grade acetonitrile (ACN) was from Fisher Scientific (Fair Lawn, NJ, USA). Mobile phases were filtered with 0.45 μM Nylon-66 filters (Fisher Scientific). All solvents were freshly prepared daily with ultra-pure grade water (electrical resistivity 18.2 MΩ·cm at 25°C).

Isopropyl-β-D-thiogalactopyranoside (IPTG, high purity grade) was obtained from Calbiochem (San Diego, California, USA) and stored in a refrigerator at 1-5°C. The samples were placed in plastic microcentrifuge tube (3000 DALTON MWCO, Millipore Corp., Bedford, MA).

Instrumentation

IC-PED was performed on a Dionex liquid chromatography system (Dionex Corporation, Sunnyvale, CA), equipped with a gold working electrode, an Ag/AgCl reference electrode. Solutions were injected with

an injection valve (Rheodyne, Inc., Cotati, CA) fitted with a 25 µL injection loop. Data collection and system control were accomplished using Peaknet software (Dionex, version 5.12) on a Dell OptiPlex Gn computer.

Separation of IPTG was achieved using an Acclaim 300 analytical column, C18, 3 µm particle size, 150 mm × 4.6 mm (Dionex). Unless otherwise specified, the mobile phase solvents were 'solvent A'=sodium acetate (NaOAc) buffer (pH 5.45, 0.01 mol/L) and 'solvent B'=acetonitrile (ACN), (A:B=90:10, v/v), delivered at a flow rate of 1.00 mL/min. All solvents were filtered, degassed, and kept under N2 (pressure about 68.95 kPa) at room temperature.

Sample preparation procedure

The rhGH sample was placed in a 1.5 mL-plastic microcentrifuge tube (3000 DALTON MWCO, Millipore Corp., Bedford, MA). The tube was microcentrifuged (fixed rotor speed 10000 r/min) for about 20 min at 4°C. After which the supernatant was discarded. A selected residual volume in the tube was weighed and diluted with degassed, deionized water at 1:1000 (v/v). This solution was then filtered through a 0.45 µm filter and injected immediately after preparation into the chromatographic system. The same amount of deionized water as the sample was used as blank solution for the same preparation process and analysis.

Results and Discussion

Electrochemical response and waveform optimization

Cyclic voltammetry has been performed for the selection of approximate IPAD waveform potentials. The current-potential (i-E) response in 0.01 mol/L pH 5.45 NaOAc buffer/ACN (90:10, v/v) degassed in the absence and presence of 100 mg/L IPTG is shown in Figure 2. Under acidic conditions, the residual response displays an anodic peak at about +1250 mV (wave a in Figure 2) during the forward scan as the formation of surface oxide takes place. On the reverse scan, a cathodic peak at about +400 mV (wave b in Figure 2) corresponds to dissolution of the surface oxide formed on the forward scan. Breakdown of solvent occurs at about +1800 mV and -1000 mV, which leads to the generation of O_2 and H_2 respectively. Reduction of dissolved O_2 takes place during both the forward and reverse scans and commences at about +200 mV.

The IPAD waveform has been shown to be effective in electronically rejecting the "background" signal from oxide formation, which is an order of magnitude larger than the signal of the analyte. The detection sequence consists of a series of triangular potential scans to maximize the signal from the transient oxide intermediates of the oxide-catalyzed mechanism. Figure 3 shows the optimized waveform for the detection of IPTG. The waveform starts at a potential lower than that required for oxide formation, scan to a maximum potential that covers oxide formation and analyte, and ends at a potential that is more negative than that required for cathodic dissolution of the formed oxide. The start and end potentials are also more positive than that required for the reduction of dissolved O_2. Hence, the contribution to the overall signal from the reduction of dissolved O_2 is minimized. On-line degassing as part of the solvent delivery system can relax constraints on the start/end potential [25]. The detection steps in both waveforms are followed by a large negative potential pulse (-2000 mV for 10 ms) to induce both cathodic cleaning of the electrode [26] and reduce any partly-soluble Au [27]. This potential pulse is necessary to extend the life of the electrode for months of continuous use. After this, a short positive potential pulse (+1600 mV for 100 ms) is used to induce anodic cleaning of the electrode. This is followed by 600 ms at -200 mV to allow for pre-adsorption of the analyte. The Johnson group has shown that amines can be pre-concentrated 10-fold with the use of an adsorption step in the potential-time waveform. PED at an Au electrode has proven to be selective for sulfur-containing compounds under mildly acidic conditions. The direct electrochemical detection of numerous polar aliphatic compounds is achieved at Au and Pt electrodes under the control of multistep potential-time waveforms. In this technique, the waveforms manage the sequential processes of sampling the faradaic signal, from the oxidation of analyte, followed by oxidative cleaning and reductive reactivation of the electrode surfaces [28]. In agreement with these findings, LaCourse and Owens [29] demonstrated that the optimum response for sulfur-containing compounds is obtained by maximizing the time of the adsorption step without negatively affecting the chromatographic integrity of the peaks.

Chromatographic conditions for IC-PAD analysis

Separation of IPTG was achieved by using an Acclaim 300 C18 analytical column with 0.01 mol/L pH 5.45 NaOAc buffer/ACN (90:10, v/v). The C18 phases have very high surface coverage, resulting in high capacity. This C18 column works at a pH between 2 and 8. Under such mildly acidic conditions, PED at am Au electrode has proven to be selective for sulfur-containing compounds [29-34]. In LaCourse's work, PED at pH 4.54 is specific to the detection of the sulfur-containing compounds IPTG. We studied the pH value of the buffer solution over the range 4-6 and found that the separation (determination) peak of IPTG is slightly variable in these mobile phases. At pH 5.45, IPTG was well separated and the corresponding response was highly sensitive. Therefore, the pH 5.45 phosphate buffer was chosen as the mobile phase. Figure 4 shows the chromatograms of IPTG standard in 0.01 mol/L NaOAc buffers at pH 5.45 and pH 4.54, respectively. IPTG was eluted at 2.87 min, with an oxygen peak observed at about 3.1 min. Dissolved O_2 was found in both mobile phases, but the presence of O_2 in the pH 5.45 solvent is a little later than that of pH 4.54 solvent. Consequently, the IPTG standard peak can be easily separated and determined at this pH value [20].

Range of linearity and limits of detection

The linear dynamic range of the IC-PED response for IPTG was determined. The response was linear over the range from 0.1 to 10 mg/L. The regression equation was Y=0.0180X+0.1267 where Y is the integral area, X is the concentration (mg/L), and the correlation coefficient (r)=0.9972 (n=7). The limit of detection (S/N=3) for 25 µL injections of IPTG was estimated to be 1 µg/L (0.1 pmol).

Application

The optimized method was utilized to determine IPTG in rhGH samples produced by Zhangjiang Bio-Tech Co. Ltd Shanghai, China. Chromatograms of rhGH products are presented in Figure 5 obtained with 0.01 mol/L pH 5.45 NaOAc buffer/ACN (90:10, v/v). The method of standard addition was performed to assay the recoveries of IPTG and the results were summarized in Table 1. Good separation and high sensitivity for IPTG were obtained.

Conclusion

Pulsed electrochemical detection following IC allows for the simple and direct detection of numerous sulfur-containing compounds. In agreement with the past efforts, IPAD is well-suited for the detection of thiocompounds such as IPTG. The IPAD waveform, which needs only to be optimized for a particular pH, allows for the direct detection of the analytes at low levels with superior limits of detection. Through the use of an acidic mobile phase (pH=5.45) under reversed-phase conditions, selectivity is achieved for IPTG. The reported method shows good stability and reproducibility. The linear ranges cover over three orders of magnitude and the limits of detection can reach 1 μg/L (0.1 pmol) for IPTG. The system performance was excellent for the rapid determination of rhGH samples. The high selectivity and good sensitivity of this approach assures that the matrix components of the assay do not interfere with the determination.

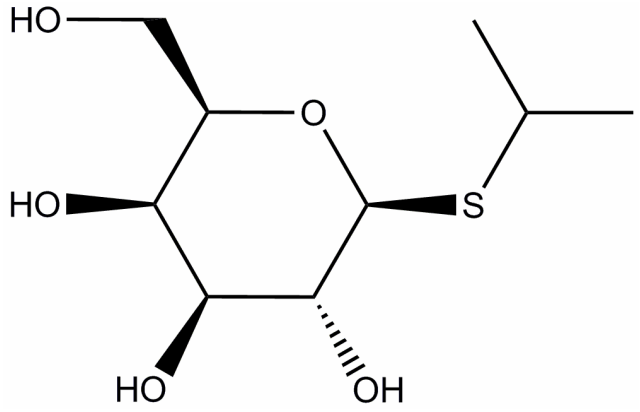

Figure 1: Structural formula of isopropyl-D-thiogalactopyranoside.

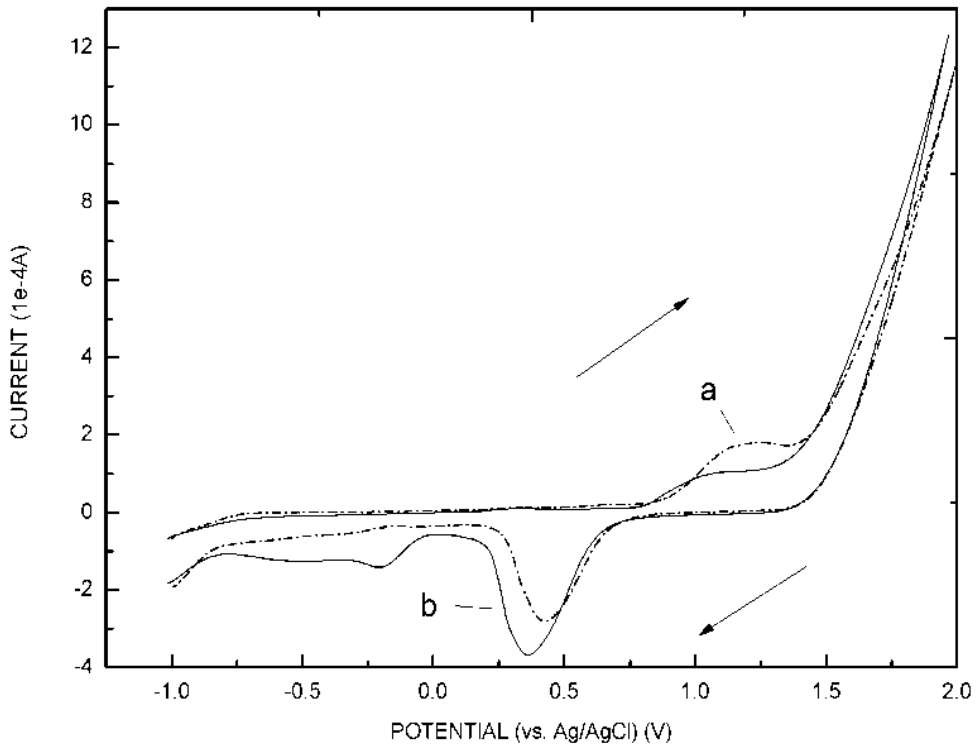

Figure 2: Voltammetric response for 100 mg/L IPTG in 0.01 mol/L pH 5.45 NaOAc/ACN (90:10, v/v) at 3 mmAu RDE. Degassed in the (b) absence and (a) presence of 100 mg/L IPTG. The forward scan (↗), the reverse scan (↙); Scan rate: 100 mV/s.

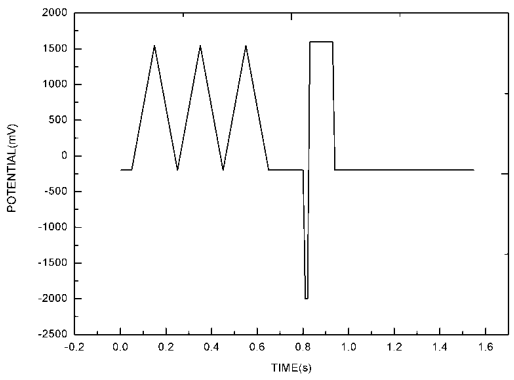

Figure 3: Waveform of the IPAD.

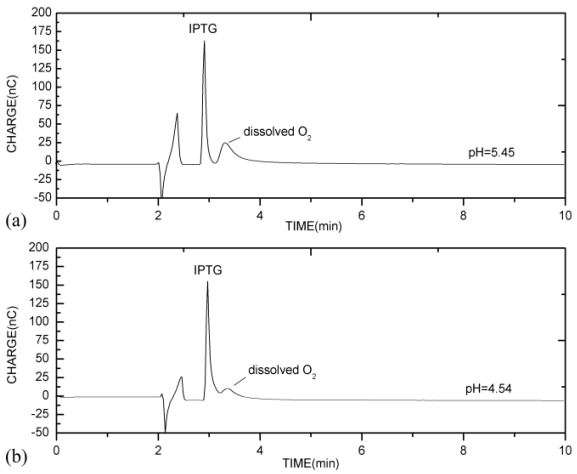

Figure 4: Chromatograms of IPTG standard in 0.01 mol/L NaOAc buffers at (a) pH 5.45 and (b) pH 4.54.

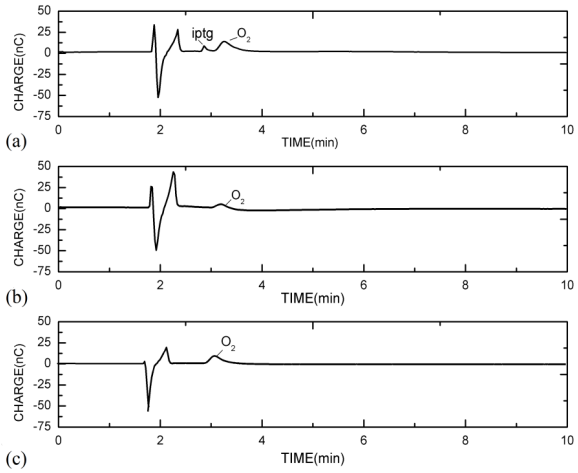

Figure 5: Chromatograms of rhGH samples. a. rhGH sample +standard; b. rhGH sample; c. blank.

Background/(mg/L)	Standard addition/(mg/L)	Found/(mg/L)	Recovery/% ± R.S.D
ND	0.020	0.020	100 ± 5
ND	0.050	0.051	102 ± 3
ND	0.100	0.100	100 ± 6

Table 1: Contents of IPTG in rhGH samples and the results of recovery test (n=3).

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