Simultaneous Enzymatic/Electrochemical Determination of Glucose and L-Glutamine in Hybridoma Media by Flow-Injection Analysis

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A split-stream flow-injection analysis system is described for simultaneous determination of glucose and L-glutamine in serum-free hybridoma bioprocess media. Amperometric measurement of glucose is based on anodic oxidation of hydrogen peroxide produced by immobilized glucose oxidase within a triple layer membrane of an integrated flow-through glucose-selective biosensor. Determination of L-glutamine is based on quantitating ammonium ions produced in a flow-through enzyme reactor containing immobilized glutaminase enzyme, and subsequent downstream potentiometric detection of these ions by a nonactin-based ion-selective polymer membrane electrode. Endogenous potassium and ammonium ion interferences in the L-glutamine determination are eliminated by using a novel in-line tubular cation-exchange membrane unit to exchange these interferent species for cations undetectable by the membrane electrode. The first generation split-stream flow-injection system can assay 12 samples/h using direct injections of 50 μL of media samples, with linear response to glucose in the range of 0.03 to 30 m*M*, and log-linear response to L-glutamine from 0.1 to 10 m*M*.

Key words: hybridoma cultivation media • glucose • L-glutamine • flow-injection analysis • biosensors

INTRODUCTION

Animal serum is often used as a supplement for mammalian cell culture because it contains a rich source of growth factors, hormones, and other nutrients. However, with the growing importance and increasing commercial use of monoclonal antibodies, the ability to grow and maintain mammalian cells in a less expensive serum-free medium, without the loss of biological productivity, has become increasingly important. In such situations, there is a need for reliable analytical monitoring systems that can detect changes in the levels of key nutrients and waste products. In principle, this information can then be used in a feedback-type control loop to maintain these critically important species at optimal levels. In low-serum or serum-free media, mammalian cells utilize both glucose and L-glutamine as energy sources and produce lactate and ammonia.[15] Thus, the monitoring of glucose and L-glutamine uptake is essential for the control of the cultivation processes.

A very promising methodology for process control in biotechnology is flow-injection analysis (FIA). Such systems consume little sample, have high sampling rates, and require relatively flexible and simple experimental setups.[17] Indeed, FIA allows the convenient combination of a variety of reagents, detection methods, and sample pretreatment devices. A growing number of studies have appeared recently regarding the application of FIA systems to bioprocess monitoring. For example, systems with chemiluminescence detection have been used for determination of glucose, lactate, lactose, galactose, and biomass.[2,10] FIA measurements with spectrophotometric detection have been employed for on-line monitoring of acetate and phosphate in cultivation processes[5] and cellulase activity in bioreactors.[23] Flow-injection amperometry with chemically modified electrodes also has been reported for determination of glucose.[1] In addition, the on-line use of flow-injection amperometry to determine monosaccharides and disaccharides, lactate, and amino acids during the production of alkaline protease and penicillin has been reported.[20] In the same work, L-glutamine was determined via spectrophotometry or gas-sensing ammonia electrode and an enzyme reactor containing immobilized glutaminase. Bio-field-effect transistors for glucose and urea; optical biosensors for the detection of ethanol, mannitol, penicillin, and urea; and a four-channel enzyme-based thermistor for the simultaneous detection of different sugars have also been reported recently for bioprocess monitoring.[18]

Several flow-injection systems have been devised for the simultaneous determination of two species in bioreactor media. Determination of glucose and ethanol with fluorometric detection has been successfully applied to food quality control and process monitoring,[12] while chemiluminescence detection of glucose and spectrophotometric detection of ammonia-nitrogen has been utilized in flow-injection monitoring in penicillin fermentations. Other significant

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developments in the application of FIA to bioreactor monitoring are provided in recent review articles.[11,19]

We recently described a novel FIA system for monitoring L-glutamine in bioreactor media.[16] This system was based on the use of immobilized glutaminase and potentiometric ion-selective electrode detection of the generated ammonia/ammonium species. Interference from endogenous potassium and ammonium ions in the media samples was eliminated by using a novel tubular ion-exchanger unit within the FIA manifold. The aim of the present study was to combine this FIA system with a glucose biosensing arrangement so that both glucose and L-glutamine can be determined simultaneously in a single injection of undiluted media from a hybridoma bioprocess.

MATERIALS AND METHODS

Reagents

For preparation of the ammonium ion-selective membrane electrode, cellulose triacetate (CTA) and nonactin were obtained from Fluka (Ronkonkoma, NY) and dipentylphthalate from Eastman Kodak (Rochester, NY).

Glutaminase (EC 3.5.1.2) grade V from *Escherichia coli,* glucose oxidase (EC 1.1.3.4) type II from *Aspergillus niger,* Iscove's Modified Dulbecco's Medium without glutamine (IMDM), and *o*-phthaldialdehyde (OPA) reagent solution for precolumn derivatization of amino acids were products of Sigma Chemical Co. (St. Louis, MO). A 5% solution of Nafion perfluorinated ion-exchanger in a mixture of aliphatic alcohols and 10% water was from Aldrich (Milwaukee, WI).

All other chemicals were reagent grade. Buffer solutions were prepared with distilled-deionized water.

Equipment

Flow-injection manifolds were assembled by using a Rainin Rabbit peristaltic pump (Woburn, MA) and a Rheodyne six-port, low-pressure rotary valve for sample injection (Cotrati, CA) with a 20- or 50-μL sample loop used for sample and standard introduction (see Fig. 1 for final dual analyte system). The in-line cation exchanger used in L-glutamine detection was a 100-cm length (0.625 mm i.d.) of Nafion 811× tubing obtained from Perma Pure Products (Toms River, NJ). This tubing was bathed in 1000 mL of lithium-acetate buffer (500 m*M*, pH 4.9) at 40°C. A twisted helix of two fishing lines was threaded through the Nafion tube in order to improve the efficiency of Donnan dialysis through the wall of the tubing.

Ammonium Sensor

Flow-through potentiometric ammonium measurements for L-glutamine determination were made with an ammonium ion-selective electrode prepared by incorporating nonactin into a plasticized (dipentylphthalate) CTA membrane[3] and then mounting a piece of this membrane into a Phillips ISE-561 electrode body (Glasblaserei Moller, Zurich). The electrode was fitted with a special cap for use as a flow-through detector in a large volume wall-jet configuration.[22] A saturated calomel reference electrode along with the working ammonium electrode (connected to the FIA system via a small length of narrow bore teflon tubing) were placed in a large beaker of reagent buffer. Potentiometric response of the working electrode was measured with an Accumet Model 910 pH/mV meter (Fisher Scientific, Romulus, MI) and recorded on a two-pen Fisher Recordall Series 5000 strip-chart recorder.

Glucose Biosensor

The flow-through glucose biosensor was made using a Corning Pt disc electrode (Model 476060; 7 mm diameter) modified by the evaporating 10 μL of a 5% Nafion solution over the surface of the platinum surface. The modified electrode surface was covered with nylon mesh with immobilized glucose oxidase (see below) and a protective Spectra/Por cellulose ester membrane with a molecular-weight cut-off of 8000 Da from Spectrum (Los Angeles, CA). The biosensor was also assembled using a special cap in a large volume wall-jet flow-through detector arrangement. The glucose biosensor fitted with this cap, along with an auxiliary Pt disc electrode and a saturated calomel reference electrode, were placed in a 300-mL beaker filled with reagent buffer. Amperometric response of the working enzyme electrode was measured with a potentiostat (Model CV-37) from Bioanalytical Systems (West Lafayette, IN) and recorded on a two-pen Fisher Recordall Series 5000 strip-chart recorder.

Preparation of Immobilized Enzymes

For L-glutamine measurements, the immobilized enzyme reactor was prepared according to a procedure reported earlier.[7] Two-hundred milligrams of controlled pore glass (170 Å, 200- to 400-mesh) from Sigma Chemical was employed for the immobilization of 50 units of glutaminase. The porous glass beads with the immobilized enzyme were then packed into a glass tube (60-mm length, 3-mm i.d.). The enzyme reactor was filled with 0.1 *M* acetate buffer, pH 4.9, and kept at 4°C when not in use.

Glucose oxidase (10,000 units) was immobilized on a nylon screen cloth (210-mesh) from Small Parts Inc. (Miami, FL) using an alkylation procedure with diethyl sulphate.[13]

Reference Methods for L-Glutamine and Glucose Determinations

Glutamine content in hybridoma media samples was determined by HPLC using the OPA precolumn derivatization method with gradient elution and fluorescence detection.[21] A Rabbit-HP system (Emeryville, CA), equipped with a

Microsorb C18 column, was utilized for all measurements. The mobile phase was a mixture of 90% sodium acetate (0.1 *M*, pH 7.2)/9.5% methanol/0.5% THF (solvent A) and 100% methanol (solvent B). The samples were diluted by 20-fold in solvent A and filtered using a 0.45-μm Acrodisc LC13PVDF syringe filters from Gelman Sciences (Ann Arbor, MI). One hundred microliters of the filtered and diluted sample was incubated with 200 μL OPA for 2 min at room temperature, and then a 25-μL portion of this reaction mixture was injected onto the HPLC column. Standard L-glutamine solutions in IMDM were used for calibration of the HPLC system.

The glucose content of hybridoma media samples was determined with a Yellow Springs Instruments Model 2000 Glucose/Lactate Analyzer (Yellow Springs, OH) calibrated with standard solutions supplied by the manufacturer. A 200-μL aliquot of the media sample was diluted with 400 μL distilled water prior to each determination.

RESULTS AND DISCUSSION

Figure 1 illustrates the final split-stream FIA system used to determine glucose and glutamine simultaneously. However, prior to assembling this arrangement, studies were undertaken to optimize the analytical performance of each of the two sensor modules of this system in a single analyte detection mode. This was accomplished by using a 20-μL sample injection and eliminating the split in the sample stream. Thus, for separate optimization of the glucose-sensing portion of the system, a 20-μL aliquot of sample was injected into a flowing water stream that was merged with a phosphate buffer (0.1 *M*, pH 6.5) stream and finally flowed through the glucose biosensor. For L-glutamine, the 20-μL sample was injected into a water stream and then merged with a stream of lithium acetate buffer (10 m*M*, pH 4.9). The diluted sample then flows through the tubular ion-exchanger unit, the glutaminase enzyme reactor, and then on to the ammonium-selective membrane electrode detector.

Optimization of Flow-Injection Amperometric Detection of Glucose

The principle of the glucose detection employed in this study is the measurement of anodic current resulting from the oxidation of hydrogen peroxide produced from the enzymatic degradation of glucose in the presence of immobilized glucose oxidase. All amperometric measurements were performed with a polarization potential of +0.65 V vs. SCE applied to the glucose working electrode. The specific design of the glucose biosensor used here evolved from the optimization of a sensor sensing reported previously for flow-injection determinations of glucose in blood serum.[8] This sensor employs a triple layer membrane. The inner Nafion film serves to prevent negatively charged electroactive species in the sample, that oxidize at the applied potential of the platinum electrode, from ever reaching the electrode. Two other layers consisted of a nylon mesh with immobilized glucose oxidase and outer protective polyester membrane. The immobilization of glucose oxidase by crosslinking directly to the inner surface of the protective polyester membrane was found to be unsatisfactory, yielding sensors with extremely low currents in response to varying glucose concentrations. Hence, the immobilization on the middle nylon mesh was used. In the optimization of the outer protective membrane, several Spectro/Por dialytic membranes of pore sizes from 500 to 8000 Da were examined. As expected, the largest amperometric response to injected glucose standards was observed with the 8000-Da cut-off membrane (i.e., larger pores yield greater flux of glucose into enzyme layer), and this outer layer was used for all subsequent studies. It should be noted that the purpose of this outer polyester membrane is prevent enzymatic degradation of the immo-

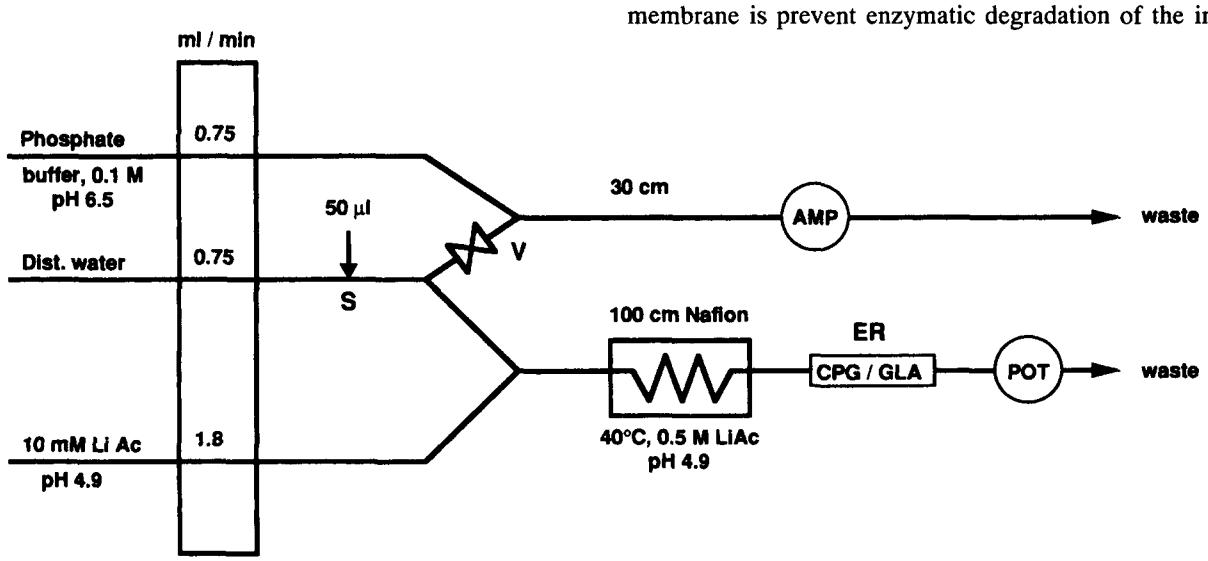

Figure 1. Schematic diagram of flow-injection system for simultaneous potentiometric determination of glutamine and amperometric determination of glucose in hybridoma media. S, sample injection point; ER, enzyme reactor with immobilized glutaminase; POT, potentiometric ammonium ISE detector; AMP, amperometric glucose biosensor; V, value.

bilized glucose oxidase (e.g., by various proteases that may be present in biological samples).

The effect of total flow rate on the flow-injection response of the glucose biosensor was examined in detail (always keeping a 1:1 flow rate between the distilled water and phosphate buffer streams). A total flow rate of 1.6 mL/min yielded the largest amperometric response to a sample containing 4 m*M* glucose. At higher flow rates, while total sample dilution is less, the magnitude of the glucose signal is limited by the response time of the biosensor. Using the 1.6 mL/min flow rate, peak currents were linearly related to glucose concentration in the range of 30 μM to 30 m*M*. A typical glucose calibration curve obtained for this flow-injection system, plotting peak height (in nA) vs. glucose concentration, is shown in Figure 2.

Flow-Injection Potentiometric Detection of L-Glutamine

One of the most convenient methods for determination of L-glutamine is the use of glutaminase and potentiometric measurement of ammonia or ammonium ions produced by the enzyme reaction. In nonflow conditions, soluble glutaminase has been used previously in conjunction with a commercial ammonia gas-sensing electrode to detect L-glutamine in bioreactor media.[14] However, to obtain accurate results, the amount of endogenous ammonia in the sample has to be determined first and then subtracted from the total ammonia detected after the enzymatic reaction.

Due to better durability, detection limits, and more rapid response times, it is more convenient to quantitate ammonia nitrogen produced by the glutaminase reaction via a flow-injection system using a nonactin-based polymer membrane ammonium ion-selective electrode as the detector. In such a scheme, however, it is necessary to eliminate or correct for interference from both endogenous ammonia/ammonium as well as certain cations, including potassium. Two different approaches have been suggested to accomplish this in our previous efforts to quantitate L-glutamine by FIA.[9,16] For the purpose of devising the proposed split-stream dual-analyte arrangement (glucose and L-glutamine), we felt it would be easier to adapt the approach in which an in-line tubular cation-exchange unit is placed between the injection valve and the downstream immobilized enzyme reactor/electrode detector.[16] In such a system, endogenous ammonium, potassium, and other interferent cations (i.e., ions toward which the nonactin membrane electrode responds significantly) are exchanged for lithium ions (which the electrode is less sensitive to) as the sample passes through the tubular ion-exchanger. L-Glutamine, being a zwitterion, remains at nearly its original concentration as the sample passes through the Nafion tube.[16] Thus, ammonium ion levels present in the sample stream after the stream passes through the reactor with immobilized glutaminase, are directly proportional to the L-glutamine concentration in the injected sample. In practice, the ability to effectively remove endogenous ions is dependent on the volume and concentration of lithium ions present in the solution bathing the Nafion ion-exchange unit. With continued use, this solution changes composition (decrease in lithium; increase in ammonium and potassium) yielding a decreased ion-exchange efficiency. Using a 1000-mL reservoir of 500 m*M* lithium acetate buffer, adequate removal of ammonium and potassium can be achieved for at least 2 days of operation.

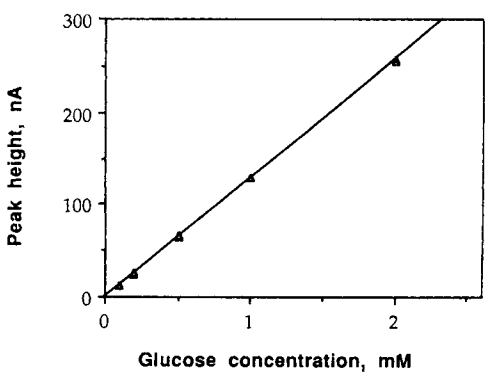

Figure 2. Typical flow-injection amperometric glucose sensor calibration plot obtained for injections of 20-μL glucose standards at total flow rate 1.6 mL/min.

For FIA L-glutamine measurements, variations in flow rate can influence the performance of several parts of the system. At very high flow rates, the residence time of the sample in the ion-exchanger unit is reduced and this leads to greater interference from endogenous cations. This point was examined in detail in our original report.[16] At the same time, a faster flow through the enzyme reactor yields lower conversion efficiency by the immobilized glutaminase, and thus reduced potentiometric signals from the downstream ammonium electrode detector are obtained. Indeed, in these preliminary studies, the efficiency of enzymatic conversion of glutamine to ammonium was estimated to be 53% using a total buffer/water flow rate of 1.3 mL/min, while at a total flow of 2.5 mL/min only 25% of the glutamine was converted. Using the slower total flow rate, very large and reproducible responses toward L-glutamine were observed, both for L-glutamine standards prepared in water and in IMDM [see Fig. 3 for calibration plots (peak height in mV vs. logarithm of L-glutamine concentration) using both matrices]. Figure 4 shows the typical calibration plots obtained using two different enzyme reactors with immobilized glutaminase. While initially, rather high slopes (e.g., typically 56 mV/decade) are observed, with continued use and further storage, the efficiency of the glutaminase reactor at higher glutamine concentrations decreases, causing a decrease in analytical sensitivity toward L-glutamine. Nonetheless, even after 2 months of use, the reactor exhibits adequate enzyme activity to be analytically useful.

It is important to note that even when using the slower flow rate (1.3 mL/min) to improve enzyme conversion efficiency and interferent ion removal, a significant signal is observed for the injection of IMDM even without glutamine added. This signal is apparently due to incomplete ion-exchange of high background sodium levels in

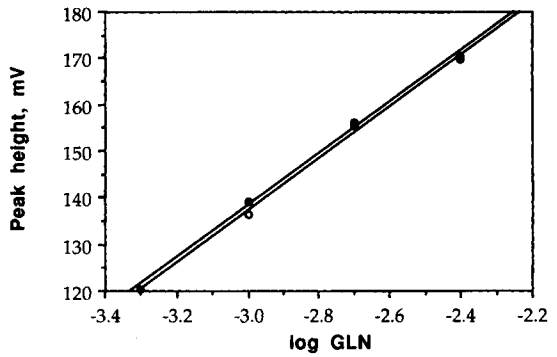

Figure 3. Flow-injection calibration plot obtained for injections (20 μL) of standard L-glutamine prepared in distilled water (O) and IMDM (●).

the IMDM. However, because the ammonium electrode responds logarithmically, this background signal does not contribute greatly to the observed responses for glutamine in the 0.5 to 4.0 m*M* range (see comparison of glutamine response in water vs. IMDM in Fig. 3). Nonetheless, in practice, more accurate values for determinations of glutamine in hybridoma media, particularly at lower glutamine concentrations (<1 m*M*) can be achieved by calibrating the FIA system with L-glutamine standards prepared in IMDM (note: the glutamine response is linear with log concentration from 0.1 to 10 m*M*).

Simultaneous Flow-Injection Determination of Glucose and L-Glutamine in Hybridoma Media

The integrated split-stream flow-injection manifold shown in Figure 1 employs the optimized flow-rate conditions found during the experiments with each single analyte FIA manifold. By controlling the flow through the restriction valve (valve V), the amount of sample entering each portion (glucose vs. glutamine) of the manifold could be maintained at the desired value. Given that a 50-μL sample injection loop is used for the simultaneous determinations, we utilized the restriction valve to split the sample stream equally between the two parts of the integrated FIA manifold so that approximately 25 μL of sample went into the glucose and L-glutamine–sensing portions of the combined system. Using this arrangement, along with IMDM solutions containing varying standard levels of glucose (20, 23, and 25 m*M*) and L-glutamine (at 0.1, 1.0, and 4.0 m*M*) for calibration purposes, we evaluated the integrated FIA system for its ability to determine both glucose and L-glutamine in various hybridoma media samples. The samples analyzed were obtained from an ongoing study relating to the optimization of transtubular bioreactors for production of monoclonal antibodies from a hybridoma cell line S3H5/2bA2 in a serum-free, low-protein media.[6]

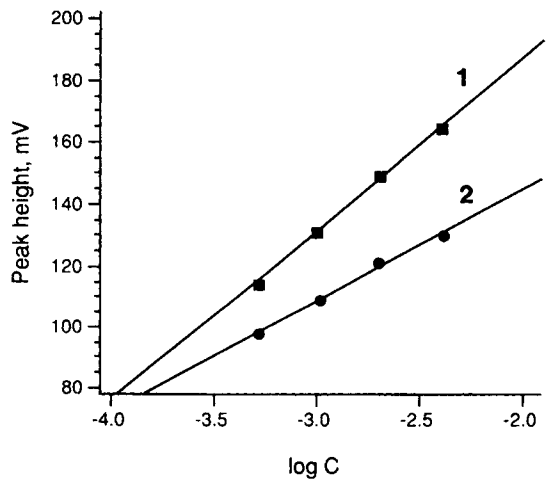

Figure 4. Calibration plots for potentiometric response in single analyte flow-injection system for L-glutamine using freshly prepared enzyme reactor (1), and enzyme reactor after 2 months of use (2).

Typical flow-injection signals from both detectors recorded simultaneously using a two-pen strip-chart recorder are shown in Figure 5 for glucose/L-glutamine standards as well as several biomedia samples (Fig. 5D–J). For 15 hybridoma samples, glucose and L-glutamine values were determined via the new combined FIA systems, and the results compared glucose levels measured using a YSI glucose/lactate analyzer and L-glutamine concentrations measured by a standard HPLC method. The correlation plot for L-glutamine values determined by the two methods yielded the following equation: $C(\text{FIA}) = 1.02^* C(\text{HPLC}) + 0.003$ with a correlation coefficient of 0.9; and, for glucose: $C(\text{FIA}) = 1.00^* C(\text{YSI}) + 0.17$ with a correlation coefficient of 0.92. Taking into account the shape of the flow-injection signal, especially for higher concentrations, the maximum sampling frequency was estimated to be 12 samples/h. Reproducibility of the signals for repeated injections of glucose/L-glutamine standards were typically <2% (r.s.d.) for each analyte.

In summary, a new split-stream biosensor-based FIA manifold system has been developed that can be used to determine glucose and L-glutamine simultaneously in hybridoma media. During 6 hours of continuous use the FIA system does not exhibit a significant change in sensitivity for either analyte; thus, relatively infrequent calibrations are required to obtain reliable results for both species. While the split-stream approach offers the simplest means to combine the two separate enzyme/electrode-based FIA systems, it may also be possible to incorporate both detection methods into a simpler single-stream FIA system. This would require, however, that the glucose detector be placed upstream from the tubular ion-exchange unit needed for accurate measurement of L-glutamine, because neutral glucose molecules can readily pass through the Nafion material.[8] Efforts to develop such a dual-analyte FIA single-stream system are currently in progress in these laboratories.

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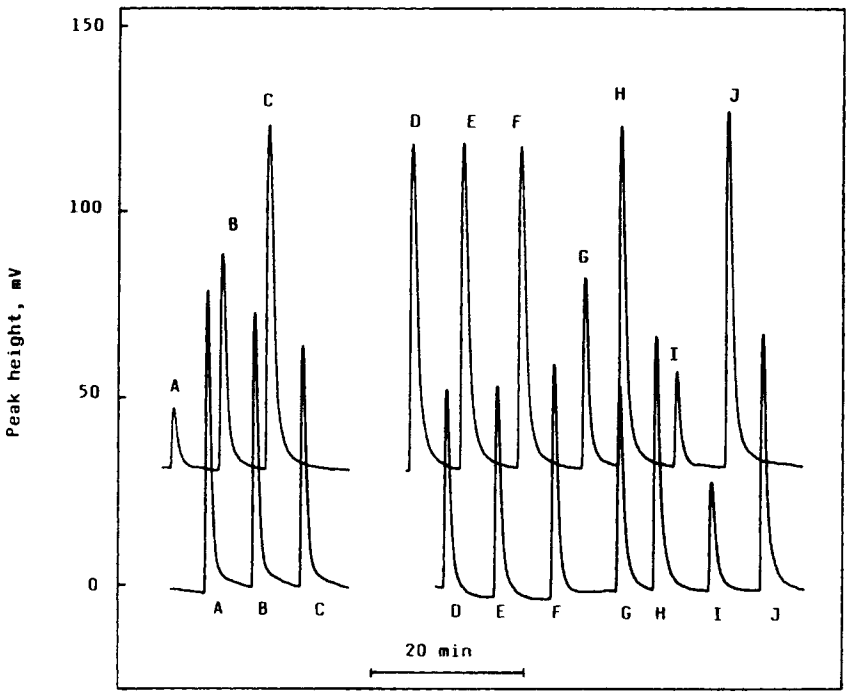

Figure 5. Typical flow-injection response recorded for simultaneous determination of glutamine (upper recording) and glucose (lower recording) in optimized FIA arrangement shown in Figure 1 for standard solutions in IMDM (A–C) and hybridoma bioprocess samples (D–J). Standard solutions contained (A) 0.1, (B) 1.0, and (C) 4.0 m*M* glutamine and (A) 25, (B) 23, and (C) 20 m*M* glucose. For glucose biosensor response, 1 mV = 45 nA.

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