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Short Communication

Enzymatic flow injection method for determination of formate*

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Introduction

Formate determination has been reported to be a useful parameter in the quality control of the sterilization of pharmaceutical preparations containing carbohydrates [1]. Chromatographic [2], optic [3], electroanalytical [4], isotacoforetic [5] and enzymatic [6] methods have been described for the analysis of formate in food, water, clinical and pharmaceutical samples. A flow injection system based on amperometric detection using a nickel electrode has been reported [7]. Enzymatic methods are based on the use of soluble formate dehydrogenase and spectrophotometric monitoring of the enzyme catalysed reaction [8].

Flow injection analysis (FIA) is characterized by a high sample throughput and easy automation [9–11] and is therefore suitable for use in quality control. The use of enzymes, that are selective (specific) reagents in FIA has extended the application of this technique to more complex samples in clinical and pharmaceutical analysis [12, 13]. Immobilization of the enzymes results in better and easier utilization of relatively expensive reagents. The usefulness of controlled pore glass (CPG) as support for immobilizing enzymes has been widely demonstrated [14]. Among the numerous techniques for the immobilizing of enzymes [15] and the different reactor configurations described in the literature [16] covalent attachment and packed bed reactors are, by far, the most widely used and advantageous procedures [14].

In this work, a sensitive and selective method to determine formate by FIA is described. The method is based on the use of immobilized formate dehydrogenase for the enzymatic oxidation of formate in the presence of nicotinamide adenine dinucleotide, NAD^+:

$$\text{formate} + NAD^+ \overset{\text{FDH}}{\rightleftarrows} CO_2 + NADH + H^+$$

A stoichiometric amount of reduced coenzyme (NADH) is then produced, being equivalent to the formate content. The NADH is subsequently detected in a UV flow through the cell at 340 nm. The flow injection method is applied to assay formic acid content in carbohydrate solutions for parenteral use.

Experimental

Reagents

Formic acid and glutaraldehyde were from Sigma (St. Louis, MO, USA). Nicotin adenin dinucleotide was from Merck (Darmstadt, FRG, Cat. No. 24542). The reduced form of the nucleotide (NADH) was from Sigma (Cat. No. N-8129). Formate dehydrogenase (FDH, EC 1.2.1.2.) from yeast was purchased as a lyophilized powder (0.53 U mg^{-1} protein); Böehringer Mannhein Biochemicals, (Cat. No. 244678, FRG) and used as received. 3-Aminopropyl-triethoxy xilane (APTX) was obtained

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from Janssen (Belgium). Other reagents were of analytical grade and Millipore/Milli-Q water was used to prepare solutions.

Immobilization procedure and enzyme reactors

FDH was immobilized on controlled pore glass (CPG-10, pore diameter 51.5 nm, particle size 37–74 μm; Serva, Cat. No. 44762) after silanization of the glass with APTX and activation with glutaraldehyde according to previously reported procedures [17]. The enzyme charged CPG was packed into plexiglas reactors with polypropylene nets at the end. The 50 μl reactor had i.d. 1.1 and length 52.6 mm. When not in use the charged CPG was stored in an 0.1 M solution of phosphate buffer (pH 7.0) at 4°C.

FIA apparatus

A diagram of the flow injection manifold is shown in Fig. 1. Carrier was delivered by a monochannel variable speed peristaltic pump (Gilson, Minipuls-2). Samples were introduced into the carrier by using a pneumatically operated injection valve (Cheminert type SVA, Kemila, Stockholm). An injection loop of 50 μl was used throughout all the experiments. The IMER was thermostatted in a water-bath. The other parts of the flow system were made of 0.5 mm i.d. PTFE tubing and the connections were made with Altex couplings. The absorbance was monitored in a 9 μl flow-through detector (Konik UV/V-200, Seattle, USA).

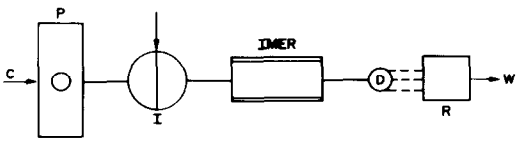

Figure 1
Complete flow system for determination of formate. C Carrier, P Pump, I Injector, IMER FDH immobilized reactor, D UV/V detector, R Recorder, W Waste.

FIA reactors (IMER)

Two different volumes of reactor were used, 50 and 400 μl. The former was used to study optimal conditions. The bigger reactor was calculated to give 99.9% conversion of formate [18] at a flow rate of 0.5 ml min^{-1}.

FIA solutions

A 2 mM NAD^+ in 0.1 M phosphate buffer (pH 7.0) at 0.5 ml min^{-1} was used as carrier solution throughout all experiments, unless otherwise stated. The concentrations of the NADH solutions, made from the commercial products, were calculated from the absorptivity of the nucleotide, 8220 l mol^{-1} cm^{-1} at 340 nm and pH 7.0.

Results and Discussion

Selectivity

Soluble FDH has been described as a specific enzyme for formate [19]. The selectivity of FDH towards acetic acid, ascorbic acid, maleic acid, oxalic acid, succinic acid and pyruvic acid was studied in a 400 μl reactor with a continuous flow of 5 mM of each compound dissolved in the carrier solution. Only pyruvic acid showed a detectable response, which represented 10% of that achieved for formate. No detectable response was found in any other of the tested compounds.

Ionic strength

The effect of the ionic strength on the immobilized enzyme was investigated by a 50 μl reactor volume and by addition of different sodium chloride concentrations into the carrier stream. 50 μl injections of 125 μM formate were made. FDH activity has been shown to be dependent on ionic strength. The results were according to Kieber *et al.* [8].

Optimal pH

The dependence of the relative activity of immobilized FDH on the pH (5.6–8.0) of the carrier was determined. An 0.1 M phosphate buffer solution containing 2 mM NAD^+ was used at a rate of 0.5 ml min^{-1}. Formate samples were dissolved into the same buffer. The activity profile shows a distinct peak around the optimal pH of 7.0.

Temperature

Optimal temperature was established by the optimal ratio between stability and activity. 40°C showed the higher activity. But an operative short life time was observed ($t_{1/2}$ = 19.2 h). The best stability:activity ratio was found at 30°C.

Flow rate

The effect of the flow rate on the conversion of formate to NADH form was investigated by using a 50 μl reactor containing the immobil-

ized FDH. The carrier was an 0.1 M phosphate buffer (pH 7.0) containing 2 mM NAD^+. The reactor was investigated in the flow injection mode with 50 μl injections of 100 μM formate at each flow rate. Samples with equal concentrations of NADH were also injected as a reference for evaluation of the conversion efficiency. At higher flow rates, there was a decrease in the conversion efficiency caused by the corresponding decrease in residence time, revealing either slow kinetic or inefficient amounts of immobilized enzyme. When these data were replotted as $[-\ln(1 - y)]$, where y is the conversion efficiency, vs the mean residence time [18], the apparent rate constant can be estimated as the slope of this plot. This constant was used to calculate the reactor volume necessary to give 99.9% conversion of formate at a flow rate of 0.5 ml min^{-1}. After these calculations a reactor volume of 400 μl was indicated to give maximum conversion efficiency under optimal conditions.

NAD^+ concentration

NAD^+ should be present in a very high concentration in relation to its Michaellis constant in order to give a pseudomonomolecular first-order reaction for formate determination. In order to establish the optimal concentration of the coenzyme, a 50 μl enzyme reactor was used; 50 μl of a 20 μM formate solution were injected into the carrier stream containing different NAD^+ concentrations 0.125–5 mM. At a higher concentration than 2 mM the conversion levels off to a constant value. No saturation was achieved in the 400 μl reactor by addition of NAD^+ concentrations up to 8 mM in the carrier stream.

Precision and linearity

The precision of the procedure was determined for different formate standard concentrations and the relative standard deviation was less than 5% in all cases ($n = 4$). The dynamic ranges observed were 5–80 μM ($r = 0.998$, $n = 9$) and 50–2000 μM ($r = 0.992$, $n = 9$) for 400 and 50 μl reactors, respectively, using an established injection volume.

Detection limit

The detection limit that can be expected with the spectrophotometric detector used is 5 μM with a signal-to-noise ratio of 3.7 (RSD ± 4.7%; $n = 4$) using 4 mM NAD^+ and 400 μl reactor.

Sample frequency

The sample frequency for the 50 μl reactor was 62 samples per hour.

Validation

The present flow injection system was applied for the determination of formate in 23 different carbohydrate pharmaceutical solutions. Each one of them was obtained in the trade (their specifications are summarized in Table 1). Three samples of each solution were introduced into the FIA system to obtain the estimates. The solutions were diluted to inject a 2% w/v carbohydrate in buffer solutions, in every case. When formate was detected, additional injections were performed using an internal standard in the same samples and so avoid the interfering effect of components in the sample matrix of pharmaceutical formulations. However, in this case such matrix

Table 1
Concentration of formate in different commercial carbohydrate solutions for parenteral use, determined through the FIA system shown in Fig. 1

Batch No.	Commercial solutions (% w/v)	Formic (μM)	Batch No.	Commercial solutions (% w/v)	Formic (μM)
1	Dextrose 5%	39.28	14	Levulose 5%	305.00
2	Dextrose 5%	n.d.	13	Dextrose 5%	289.00
3	Mannitol 20%	n.d.	15	Dextrose 10%	n.d.
4	Dextrose 5%	115.78	16	Dextrose 10%	n.d.
5	Dextrose 5%	221.42	17	Levulose 10%	1,762.00
6	Dextrose 5%	35.70	18	Mannitol 10%	n.d.
7	Dextrose 5%	36.60	19	Dextrose 20%	n.d.
8	Dextrose 5%	39.60	20	Dextrose 30%	206.50
9	Dextrose 5%	102.90	21	Dextrose 30%	191.40
10	Dextrose 5%	46.80	22	Dextrose 40%	625.00
11	Dextrose 5%	15.00	23	Dextrose 50%	1,467.40
12	Dextrose 5%	26.00			

effects were not observed. A summary of the analysis of selected samples is given in Table 1.

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