Automated Flow Injection Method for the Assay of Xanthine Oxidase Activity

M. MASOOM
Institute of Biochemistry, University of Baluchistan, Quetta-Pakistan

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Summary: An automated flow injection system is described, based on the principle of stopped flow-merging zones, for the estimation of xanthine oxidase activity. The method is based on monitoring spectrophotometrically the production of uric acid at 290 nm. Hypoxanthine is used as a substrate for the enzyme. A good linear calibration with a correlation coefficient of 0.9928 for xanthine oxidase standards (0-100 U/L) is obtained.
The precision is 2.0% and sample throughput is 35-40 /h.

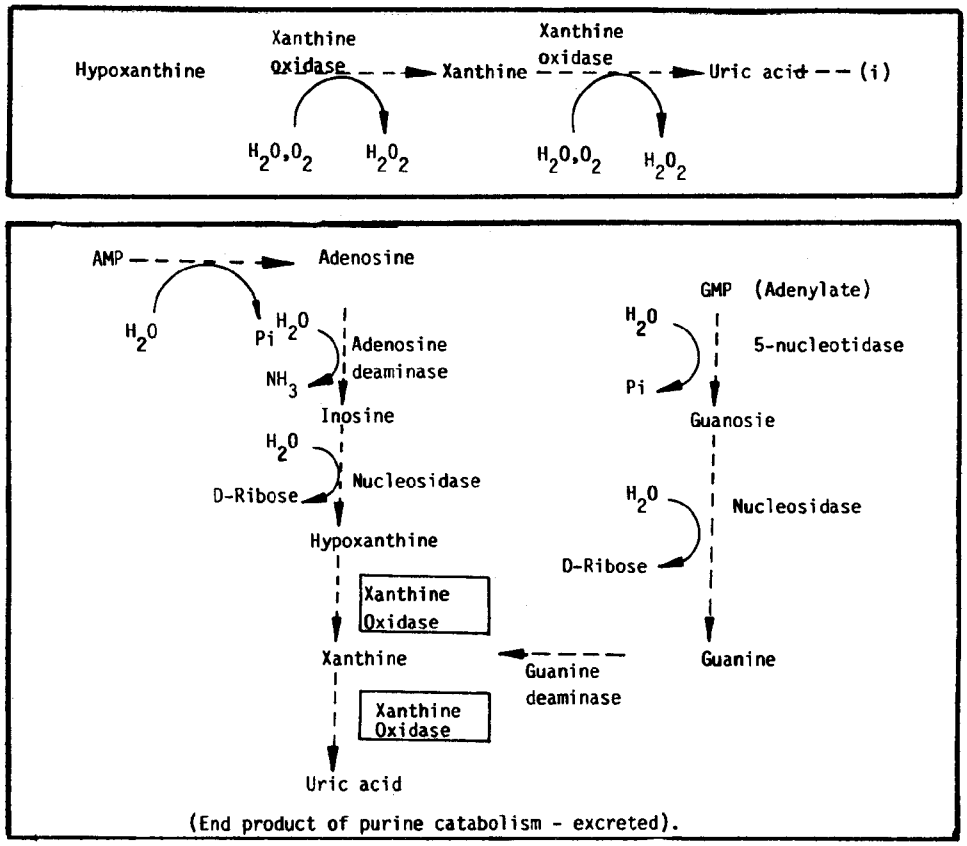
Introduction

Xanthine oxidase (xanthine: oxygen oxidoreductase, EC 1.2.3.2.) is a metal flavoprotein containing FAD, molybdenum and iron in the ratio of 2:2:8 [1]. Xanthine oxidase exhibits low substrate specificity, thus reacting with various classes of substrates, but its main role is to oxidise hypoxanthine and xanthine to uric acid with the most rapid rates and the lowest 'Km' values [2].

The catalyzed reaction is:

Xanthine oxidase is the last enzyme in the pathway for the degradation of purine derivatives and therefore the rate limiting step in purine metabolism.

Xanthine oxidase is an intracellular enzyme of mammalian tissue confined mainly to the liver cells [3]. Serum xanthine oxidase activity is exclusively dependent on the production of the liver, thereby markedly increasing the serum enzyme level in



patients with viral hepatitis [4]. Thus serum enzyme level plays a key role in the differential diagnosis of the jaundice [5]. Various methods have been used for the estimation of xanthine oxidase activity: e.g. manometrically [6] colorimetrically [7], fluorimetrically [8] and with isotopic method [9]. But the most usual method is a spectrophotometric one which involves monitoring the production of uric acid at 293 nm during the course of oxidation of hypoxanthine or xanthine to uric acid by xanthine oxidase [10]. The same method has been adopted in the present investigation using an automated flow injection system. The system is very flexible and provides a direct, sensitive and rapid method for the assay of xanthine oxidase.

Experimental

Reagents

Xanthine oxidase (xanthine; oxygen oxidoreductase, EC.1.2.3.2.) was obtained from Sigma London. Standards covering the range 0- 100 U/L were prepared in deionized water. Hypoxanthine was also obtained from Sigma London. A stock solution of hypoxanthine (1.0 mmol/L) was prepared in Tris buffer 0.1M, pH9.0). The working solutions of the substrate were prepared by diluting the stock solution.

Instrumentation and Procedure

A schematic representation of the flow injection manifold used is shown in Fig.1, which was automated using a microcomputer (BBC model B) as described previously [11]. The detector used was a single beam spectrophotometer (LKB, Ultrospec.) equipped with a 8 ul flow cell. The computer reads the absorbance values directly from the spectrophotometer into the software. Detailed computer programme used as system software is given in the appendix. Enzyme standard solutions' sample (20 ul) and substrate solutions (20 ul) were simultaneously injected into Tris buffer (0.1, pH 9.0), with a total overall flow rate of 2.5 ml/min for both the channels using a peristaltic pump (Ismatic mini S820). The pumping rate being controlled by polyvinyl pump tubing. Polyethylene tubing (internal diameter 0.5 mm) was used to connect the various components throughout the system. The distance from the confluence point to the detector was 40 cm (the smallest possible distance), inclusive of a packed reactor (2.5 * 25 mm) containing glass beads to ensure complete mixing of enzyme and substrate. The enzyme and substrate mixed zone was stopped in the flow cell for 120 s and the absorbance was monitored at 290 nm. All readings were taken in triplicate.

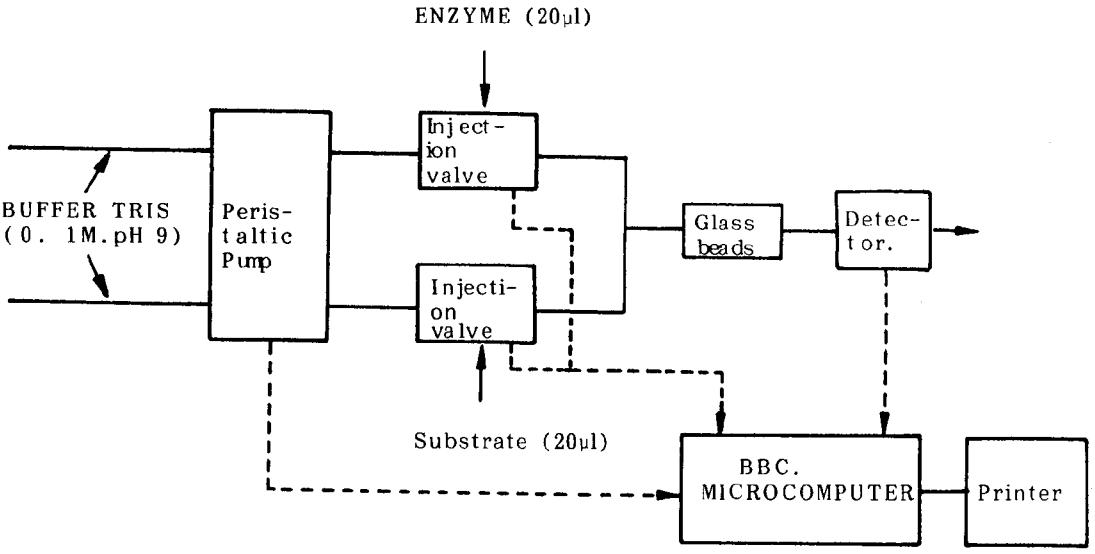


Figure 1.

Results and Discussions

Optimization of conditions for xanthine oxidase assay

The effect of pH on the rate of reaction was investigated by using Tris buffer (0.1M) of various pH values as the carrier stream. Using 50 mM hypoxanthine concentration as the standard and the 20 U/L xanthine oxidase, the maximum rate was obtained at pH 9.0 (Fig. 2), which was used for all further experiments.

The optimum substrate concentration for the assay was selected by taking various concentrations of hypoxanthine (1.0 - 100 mM) by diluting the stock solution. The enzyme concentration was 80 U/L and the pH of the buffer was kept 9.0. The highest rate was obtained at 50 mM hypoxanthine (Fig. 3). Therefore solution of 50 mM hypoxanthine was injected simultaneously with the enzyme for assay studies.

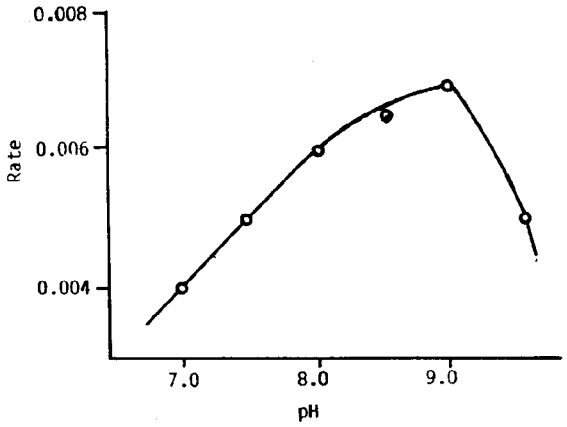


Figure 2.

Calibration for Xanthine Oxidase

Six xanthine oxidase standards (0-100 U/L) were injected into the flow injection manifold shown in Fig. 1, and analysed. As shown in Fig. 4, a good linear calibration is obtained. The within batch precision for the 60 U/L xanthine oxidase standard, analysed ten times was $\pm 2\%$.

The total analysis time was 85 s per sample, thus giving a sample throughput of 35-40/h.

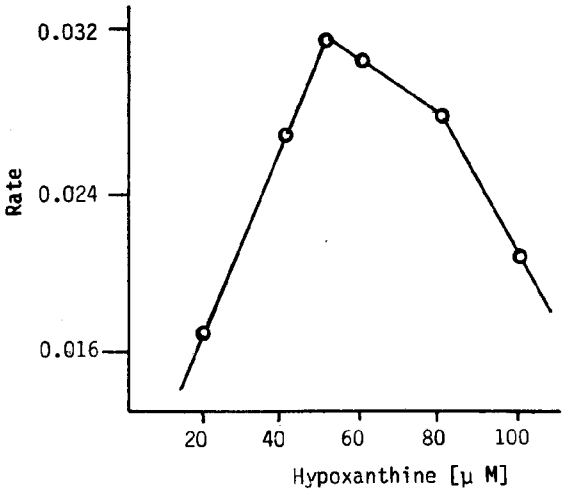


Figure 3.

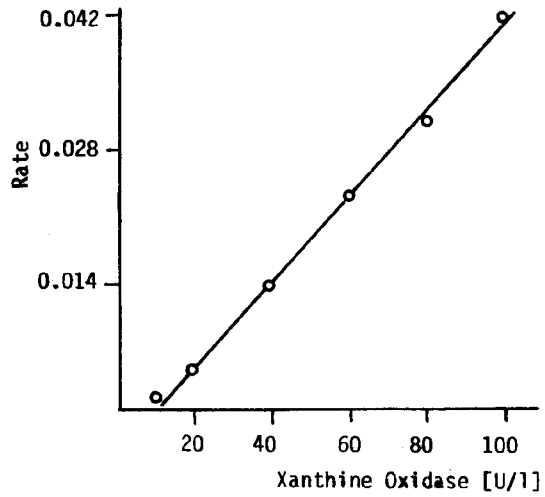


Figure 4.

Conclusion

The precision, accuracy and sensitivity of this low cost, automated flow injection system is found to be satisfactory for the rapid and reliable determination of enzyme activity in a clinical laboratory.

References

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