Automated Fluorimetric Determination of Creatine Using Flow Injection Analysis:

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Summary: An automated flow-injection method for the determination of creatine by a stopped flow procedure is described. The response used is the rate of development of fluorescence when creatine reacts with ninhydrin in alkali. The linear range, precision and accuracy of the technique for creatine is given. The advantages of this approach to the determination of creatine in serum, blood and urine is also discussed.

Introduction

Flow injection analysis (FIA) has the advantages of high sampling rate, low reagent consumption, negligible carryover, ease in automation and also simplicity and versatility[1]. The additional application modes developed for basic FIA, such as the principle of merging zones[2] has further explored the versatility of the technique. Since its emergence in 1975, the technique has been used for the analysis of hundreds of substances[3]. This report also describes such an application of

automated merging zone flow injection system using a fluorimetric detector. Creatinuria or excessive excretion of creatine in the urine occurs in many conditions, mainly hyperthyroidism, some muscle diseases and in rheumatoid arthritis[4,5]. Furthermore creatine is liberated in the assay reaction of creatine phosphokinase[6]. Therefore the present system can be linked with creatine phosphokinase (CPK) reaction for the assay of CPK, an important enzyme in the diagnosis of myocardial infarction[7].

The most widely used methods for determining creatine are based upon the conversion of creatine to creatinine by heating in the presence of strong mineral acids and subsequent determination of the creatinine by Jaffe's reaction[8]. Diacetyl reaction for the estimation of creatine has also been used[9]. Conn and Davis [10] found that in strongly alkaline solution, ninhydrin combines with creatine to give a strongly fluorescent product.

Creatine + Ninhydrin $\xrightarrow{KOH}$ Fluorescent product.

This reaction scheme has been adopted in the present investigation. The method is simple, convenient and has a high degree of precision.

Experimental

Reagents:

Creatine was obtained from BDH chemicals, and further purified by recrystallization with warm water. 1% stock solution of creatine hydrate was prepared in deionized water. Standards covering the range 0-100 μg/l were prepared from stock creatine solution. Ninhydrin which was used as a reagent was also obtained from BDH. A 10% (W/v) ethanolic solution of this reagent was prepared by dissolving 10 g of ninhydrin in 50 ml of 95% ethanol and making to 100ml with deionized water. The stock solution was then diluted to give 0.5, 1.0, 2.0, 3.0, 4.0, 5.0 and 6.0 per cent solution of ninhydrin.The effect of ninhydrin concentration on rate of change in fluorescence was determined using 40μg/l creatine standard (for method see later). A graph of rate of fluorescence development against ninhdyrin concentration showed a plateau at a concentration of 5%, so this concentration of ninhydrin was used in further experiments. Potassium hydroxide used was also of Analar grade.A 10% ethanolic solution of KOH was prepared by dissolving 10 g KOH in 10 ml of 95% ethanol and making to 100 ml with deionized water.

Instrumentation and Procedure

The flow injection mainfold used is shown in Fig.1. Creatine standards (20μl) and ninhydrin (20μl) were simultaneously injected into separate carrier streams using two low pressure injection valves (Rheodyne 5020) and synchronously merged 12 cm downstream. The carrier streams of deionized water were each pumped at 2.5 ml/min, using polyvinylchloride pump tubing and a peristaltic pump (Ismatec mini S820). Teflon tubing (0.5 mm i.d.) was used throughout the remainder of the system.The distance from the confluence point to the detector was 50 cm. This included the introduction of a third stream (of KOH) also at 2.5 ml/min. to the merged zone and then a packed reactor (2.5 cm

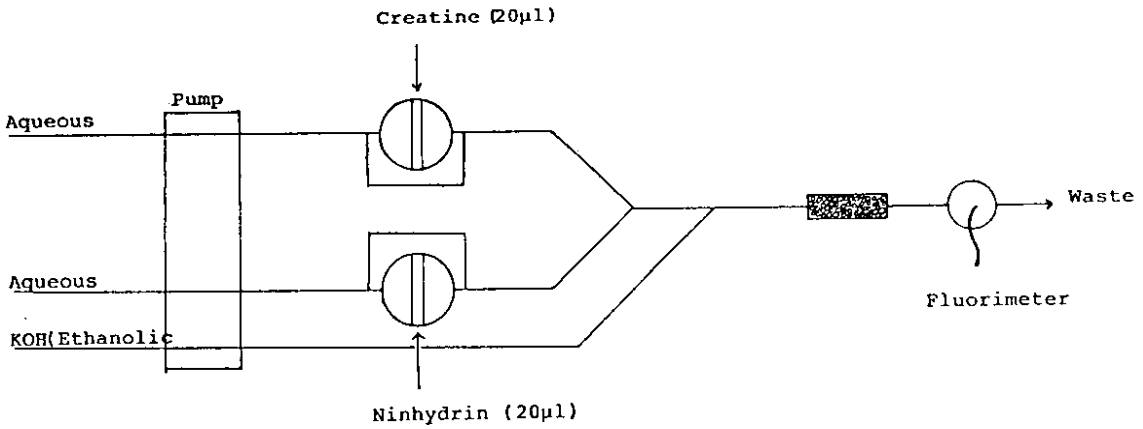

Fig. 1: Stopped Flow merging zones manfold for the fluorimetric determination of creatine.

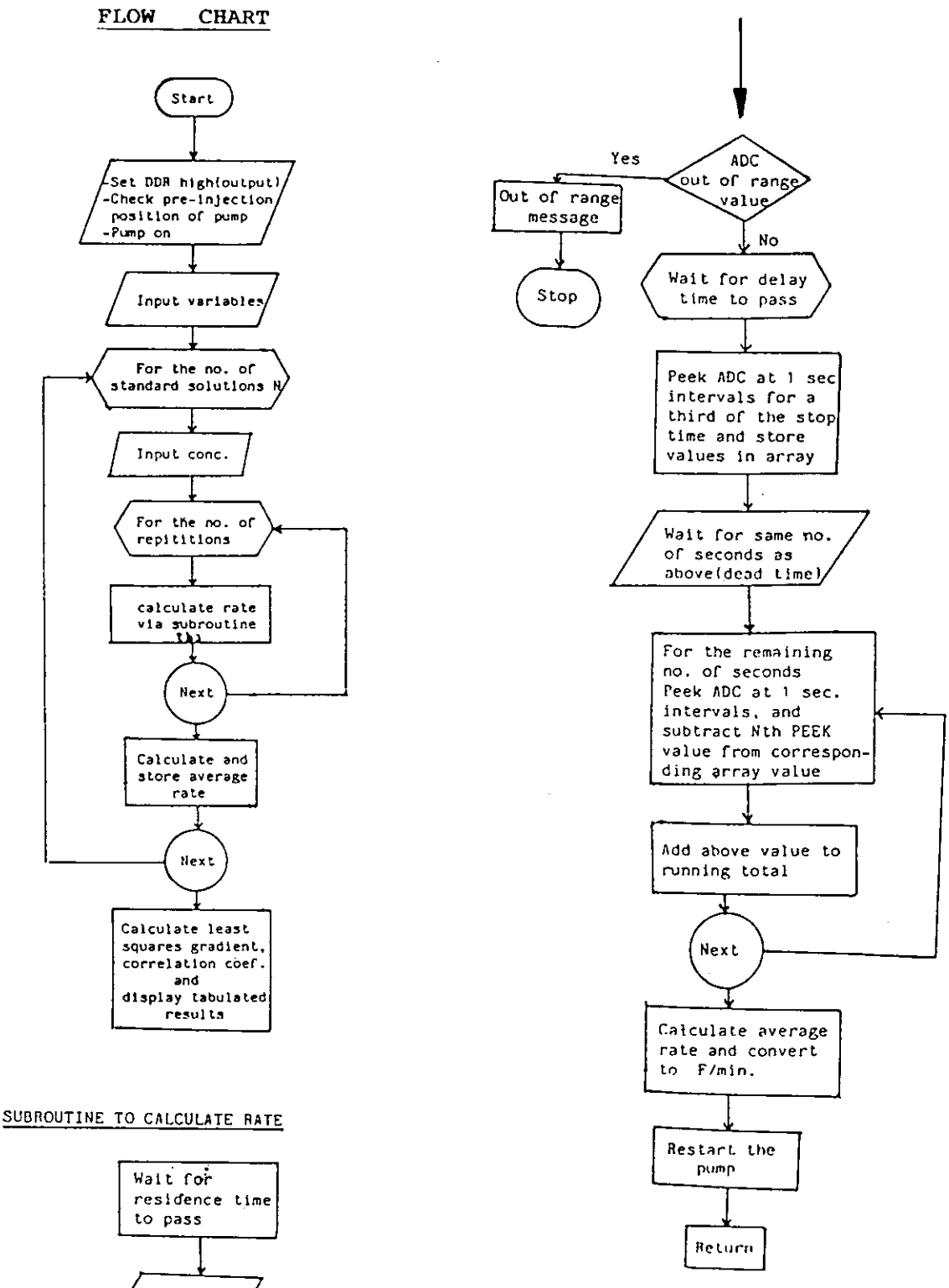

Fig. 2: Flow chart of the automated system software.

x 1.5 mm i.d.) containing glass beads (0.5 - 0.75 mm diameter) to ensure complete mixing of sample and reagents. The detector was a filter spectrofluorimeter (Perkin Elmer LS2) equipped with a 7 μl flow- through cell, and the excitation and emission wavelengths were 400 nm (filter) and 500 nm respectively. The signal from the spectrofluorimeter (1 volt full scale) was fed directly to the analogue to digital (A/D) converter of a microcomputer (BBC model B), at a rate of one reading per second, with 10 bit resolution.

The rate of reaction was determined by stopping a segment of the merged sample and reagent zone in the flow-cell and performing a moving point kinetic analysis. This was achieved by switching off the pump 14.5s after sample injection, waiting 10 second for the reaction zone to settle in the flow cell and then taking fluorescence readings during a predetermined measurement time, which in this case was 60 seconds. The measurement time was divided into three equal periods and readings were taken at the rate of one per second over the first and third periods. A set of reaction rates was then obtained by difference and the mean reaction rate determined.A flow chart of the system soft ware is given in Fig.2, which explains the functioning and operation of the system. All results reported are from triplicate injections.

A microcomputer (BBC model B) was used to control the operation of the two injection valves, the switching of the peristaltic pump, the reproducible timing of events and the collection and treatment of data. The timing was controlled by an on-board lapsed time clock, reproducible to within 0.01s. The switching of the valves and the pump was controlled by three optically isolated a.c. output modules (Radio Spares 348-469), capable of switching output loads operating on a.c. supplies between 48 and 240V a.c., at currents upto 3A, from a T.T.L. Open collector compatible input signal. The a.c. modules were connected to the user port of the BBC via a 7407 hexadecimal driver chip (RS 75492) to protect the user port from reverse voltages. Two injection valves were connected to a modified pneumatic actuator (Rheodyne 5701) such that simultaneous injection was achieved when the actuator was activated. The pneumatic arm was driven by compressed air (60 p.s.i.) via two solenoid valves (RS 348-380) connected to two a.c. output modules. The third a.c. output module was used to control the peristaltic pump.

A block diagram of the automated flow injection system is shown in Fig.3.

Results and Discussion

Determination of Creatine:

The reaction and the rate measurement conditions were optimized first. As described earlier, the optimum concentration of ninhydrin was first found to be a 5% ethanolic solution of ninhydrin. The residence time of the sample-reagent merged zone was then worked out. Nine creatine standards were then injected into the manifold shown in

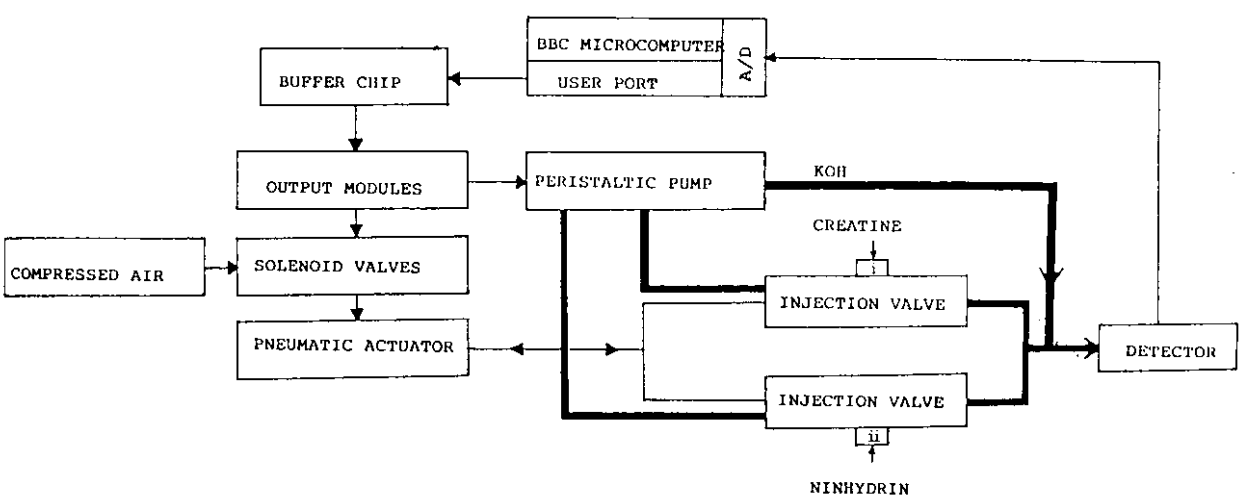

Fig. 3: Block diagram of the automated flow injection system.

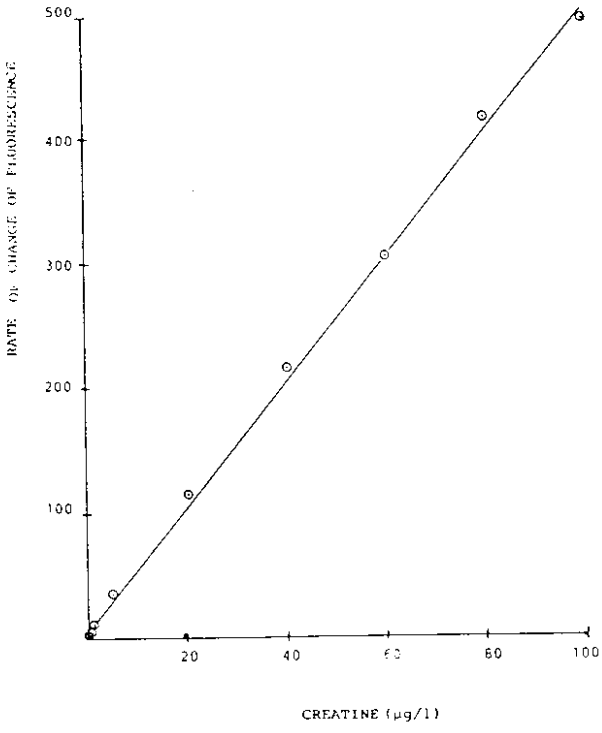

Fig. 4: Calibration graph for creatine.

figure 1. The rate of the formation of product with increasing creatine concentration gave a very linear calibration graph for 0-100µg/l creatine (Fig.4.) with a correlation coefficient of 0.9983. The within batch precision for the 40µg/l standard, analysed ten times was 2.3%. The precision in merging zone flow injection kinetic analysis is governed by the exact and reproducible merging of the sample and reagent as well as the reproducible residence time of the merged zone. The small value of r.s.d. (2.3%) suggests both these parameters to be very reproducible. The total analysis time ws 85s per sample, giving a realistic sample throughput of 35-40h^{-1}.

Conclusions

This paper demonstrates the well documented attractions of FIA, namely high throughput, rapid readout, flexibility, low cost and ease of automation. This method can not only prove useful in the estimation of creatine in biological fluids but also to other substances measurement in biological fluids which may involve such a kinetic study. Of particular importance for the determination of these substances such as metabolites and plasma enzyme levels are the small sample and reagent volume required in the merging zones manifold and the sensitivity achieved by the stopped-flow procedure with fluorescence detection.

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