

Kinetic Determination of Uric Acid in Urine Based on Single-Line Flow-System with Multi-Site Detection

Alberto N. ARAÚJO[†], José A. M. CATITA and José L. F. C. LIMA

*CEQUP/Dep. Química-Física, Faculdade de Farmácia, Universidade do Porto,
Rua Aníbal Cunha 164, 4050 Porto, Portugal*

A flow injection system based on multi-site detection for kinetic spectrophotometric determination of uric acid in urine is described. The system was studied regarding physical dispersion, elimination of sample matrix interferences and applicability to real samples. In the optimized system, the sample is inserted into a water stream which merged downstream with the reagent solution containing iron(II) chloride and TPTZ. This system enables the determination of about 30 samples/h, and yields precise results (RSD usually <2.9%). Sensitivity is 212 mAU l/mmol up to 0.60 mmol/l uric acid. Accuracy was assessed by running 32 samples already analyzed by a conventional enzymatic procedure. No statistical difference between methods was found at the 95% confidence level.

Keywords Multi-site detection, continuous flow kinetic determination, uric acid, urine

Kinetic evaluation of a reagent amount in chemical determinations has been used to quantify low-concentration catalytic species or to improve selectivity of these procedures.^{1,2} When kinetic determinations are performed, the time period for the reaction evaluation must be carefully controlled as it may affect the quality of results. Its automation thus becomes essential. Flow injection analysis is well suited to accomplish this task as it is based on reproducible timing phenomena. The proposed analytical set-ups with two serial detectors³, sample double injection⁴, flow splitting and confluence⁵, stopped flow mode⁶ and sample zone recirculation⁷, have proved the usefulness of continuous flow-injection as a versatile procedure of performing kinetic determinations. The systems resorting to more than one detector along the tubular path through which the sample flows³ or the injection of several aliquots at different distances from the detector⁴ are the easiest to implement and handle in relation to the aforementioned procedures. However, both arrangements may be cumbersome when economic aspects and sample source have to be considered.

Multi-site detection⁸ is based on the location of the flow-through cell of a detector at the loop of an injection device. According to this principle, a detection flow through cell can be displaced between two points of the manifold. Therefore, one can monitor different parameters in two parallel channels^{8,9}, avoid the passage of air bubbles into the detection system in mono-segmented streams^{10,11} and reduce the time spent in washing the detector flow cell after the zone sample flow.¹²

The displacement of the detector flow cell between two points of a tubular path to achieve kinetic determinations is reported in this work. Determination of uric acid based on its reducing effect on iron(III) is implemented. In this slow reaction, the amount of iron(II) formed is colorimetrically monitored after the colored complex with tripyridiltriazine (TPTZ) is formed.¹³ The obtained analytical signals were processed in order to compensate physical dispersion derived from the continuous nature of sample flow. The elimination of sample matrix interferences was also assessed by studying the influence of ascorbic acid as fast reducing agent of iron(III). Finally, the quality of the results given by the proposed methodology was assessed by performing uric acid determinations in urine.

Experimental

Reagents and solutions

Analytical grade reagents and de-ionized water (conductivity <1 µS/cm) were used for the preparation of the different solutions.

A reagent stock solution was prepared by dissolving sequentially 16.8 g sodium hydroxide, 5.0 g Brij 99, 526.0 mg iron(II) chloride and 500.0 mg TPTZ in about 800 ml ethanol (90% v/v). Afterwards, 30 ml glacial acetic acid were added to this solution and its volume made up to 1 l with ethanol (90% v/v). This solution was kept from light at 4°C, remaining stable for one year.¹³ The working reagent solution was daily prepared by mixing one volume of the stock solution with 2.5 volumes of water; this solution was kept away from light.

[†] To whom correspondence should be addressed.

A stock solution of 6.0 mmol/l uric acid was prepared each week by dissolving 100.0 mg uric acid in about a 15 ml solution of 4 g/l lithium carbonate heated at 60°C. Afterwards, this solution was made up to 100 ml with water and kept away from light at 4°C. Calibrating solutions in concentrations of 0.12, 0.24, 0.36, 0.48 and 0.60 mmol/l, respectively, were daily prepared from this stock solution by careful dilution.

Ascorbic acid aqueous solutions in concentrations of 0.06, 0.12, 0.17 and 0.23 mmol/l were used to study its interference effect.

The quality of the results provided by the present methodology was assessed by comparison with the results given for the same samples by the enzymatic method¹⁶, commercially available as ready-to-use reagent: Acide urique enzymatique PAP 150 (bioMérieux®, Marcy l'Etoile, France). Urine samples were stored in a refrigerator before use.

Apparatus

A home-made commutator¹⁴ with two intercalation sections was used in the developed manifold (Fig. 1). The two ports of the mobile slide corresponding to the first intercalation section were connected by means of a piece of 0.8 mm i.d. tubing; this created the sample injection loop. Both mobile slide ports of the second insertion point were connected by a Hellma 178.712 QS flow-cell of 18 µl optical volume. This cell was held by a plastic support linked to two optical fibers which were lined up with the optical path. The measurement of the analytical signal was carried out by a modular colorimeter device consisting of a LED based multi-color source World Precision Instruments (WPI), Model FOCW (Sarasota, Florida), and a photodiode WPI detector, Model VISD (Sarasota, Florida), coupled

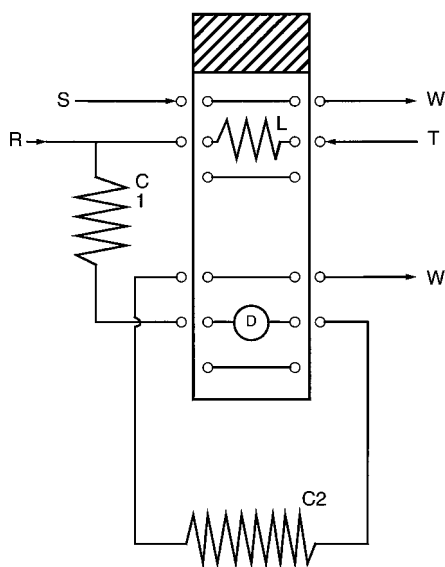


Fig. 1 Schematic representation of the proposed manifold: T, water; C1&C2, coiled reactors; D, detector; L, sample loop; W, waste; R, reagent; S, sample.

to a WPI amplifier, Model PDA1 (Sarasota, Florida). The analytical signals were recorded by a Kipp & Zonen 111 BD recorder. As the analytical signal transmitted by the amplifier was in direct proportion to the intensity of radiation, a home-made logarithm/linear converter was placed between the amplifier and the recorder so that signals proportional to absorbance could be obtained.

Solutions were propelled by a Gilson Minipuls 2 peristaltic pump, equipped with PVC tubing of the same brand. PTFE tubing (0.8 mm i.d.) was used to connect the different components of the system.

Determinations of uric acid by the enzymatic method were carried out by using a double beam spectrophotometer (Hitachi U2000, Tokyo, Japan).

Methods

In the analytical set-up, a sample volume was inserted into a water stream which merged with the color developing reagent. The displacement of the commutator mobile slide assured the positioning of the detector at the end of reactor C1, as well as the sample intercalation. The mobile slide was kept in this position over the time interval required for the sample plug to cover coil C1 and flow through the detector (Fig. 2A); the first transient signal was thus obtained (Fig. 2B, H1). Afterwards, the slide was displaced to its former position, what enabled one to fill up the first intercalation

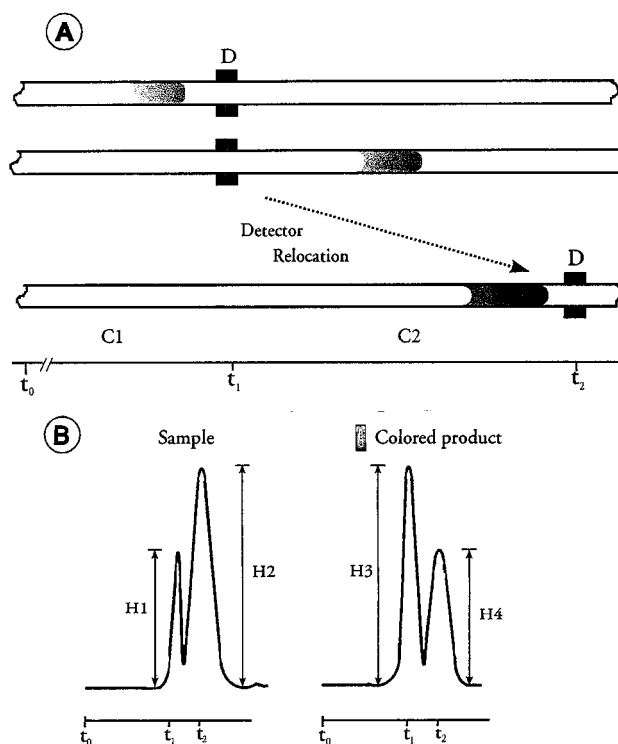


Fig. 2 A) Detail of the multi-site detection behavior: t_0 , injection time; t_1 , 1st detection time; t_2 , 2nd detection time. B) Typical outline of the analytical signals obtained by insertion of uric acid calibrating solutions and colored product solutions.

section loop with a new sample and to locate the detector at the end of C2, simultaneously. When the sample plug flowed again through the detector after having covered C2, it provided a second transient signal (Fig. 2B, H2).

Physical dispersion of the intercalated sample plug was evaluated by injecting solutions in different concentrations of Fe-TPTZ colored complex. Therefore, 15 mg TPTZ were dissolved in alcohol and made up to 100 ml with a 10^{-5} mol/l HCl solution. Small amounts of Fe(II) solution were slowly added to this mixture and the color development was monitored until its absorbance at 593 nm was found to be stable. This solution was diluted with water to prepare solutions with different color intensities.

The uric acid concentration of the samples analyzed according to the proposed method was determined previously by the enzymatic method.¹⁵ This method was carried out by adding in a test-tube, 20 μ l of ten fold diluted sample and 1000 μ l of reagent. After a 10 min incubation period at room temperature, absorbance at 520 nm was measured. Uric acid concentration was finally determined by graphic interpolation in a calibration curve, which had been constructed with the absorbance values obtained in batch conditions for the calibrating solutions used in the proposed methodology.

Results and Discussion

The intercalation of a reduced volume of a sample into a flowing stream of liquid, as it occurs in flow injection conditions, originates the gradual and controlled dilution of the former in the latter. This dilution depends on the residence time, reactor geometry and injected volume. The residence time, *i.e.* time interval between sample injection and the maximum analytical signal recording¹⁶, can be changed by setting the flow rate and varying the length of the tubular path from the injection unit up to the detector or *vice versa*. This feature was initially studied by setting the injection volume at about 30 μ l and varying in turn the flow rate from 1.44 to 3.12 ml/min as well as the lengths of reactors C1 and C2 from 120 to 270 and 150 to 350 cm, respectively. The injection of reaction colored product solutions revealed similar analytical signals for the same residence time, regardless of the possible manifold arrangements for the studied variables. Hence, it was decided to fix the flow rate after the confluence to 2.28 ml/min. This allowed us to change freely the residence time of the sample in C1 and C2, by changing their length.

With the length ranges of C1 and C2 given before, the insertion of solutions with uric acid concentrations less than 0.12 mmol/l produced larger and less intense signals when the detector was placed at the end of C2 in relation to those obtained when the detector was located at the end of C1. This effect caused by the sample dilution between the detection sites was more obvious

when the length of C2 was increased. The relationship between the sample dilution level and the residence time regarding tubular paths with different tridimensional configuration was already described.¹⁷ The dilution level herein was assessed by the dispersion coefficient, defined as the ratio of the analytical signal intensity of the solution before and after its injection into the proposed system¹⁸, and it was found that the knitted open tubular (KOT) reactors experienced a lower dilution for the same residence time. Nevertheless, it was also observed that even when C1 and C2 were KOT reactors, lower transient signals were provided at the second detection site comparing with those given by the first. The physical dispersion of the proposed system was then evaluated and the relationship between sample dispersion and injection volume, or C1 and C2 length, was assessed after injection of different colored product solutions having a color intensity in proportion to its concentration. It was found that the dispersion coefficient decreased in both detection sites significantly when the volume inserted was increased (Fig. 3). The variation of the dispersion coefficient was more evident when injection volumes of less than 30 μ l were used. This was caused by the closer proximity of both interfaces of the sample to the carrier, which promoted the dilution. A constant value of 1.43 ± 0.03 for the dispersion rate (DR), expressed as the ratio of intensity of the first (H3) and second signal (H4) (Fig. 2B), was obtained. It proved that the relative increase of dispersion between both detection sites was constant and not related to the volume or the concentration of the colored product inserted. The product between DR and H2 allowed one to estimate the intensity of the second analytical signal and to correct it for the dilution effect.

The time interval between the two detections was also increased (20 to 55 s) by means of lengthening C2 from 100 to 350 cm, to determine its influence. It was

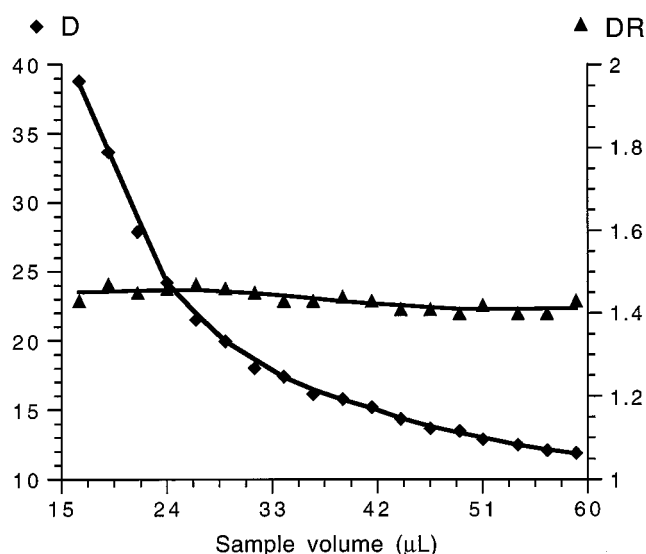


Fig. 3 Effect of the sample volume on dispersion coefficient (D) and dispersion rate (DR).

found that DR increased from 1.23 to 1.62 because of the increase of C2 length. Finally, the first detection time was varied from 22 s to 59 s by increasing C1 length from 100 to 320 cm, and a diminishing of DR (17%) was observed. This can be explained by the gradual diminishing of the RSD of the dispersion caused by the lengthening of the flowing sample zone.

Uric acid calibrating solutions were inserted under the same conditions used for the studies of dispersion previously described. The variation of intensity of the analytical signals produced by the uric acid concentration was plotted and it showed a non-linear outline. As already mentioned, this derived from the greater influence of the dispersion effect over the rate of the colored product formation. However, plotting the difference between the intensity of the second analytical signal (H2) achieved by the dispersion rate (DR) and that of the first signal (H1) as a function of uric acid concentration, it was found that there was a linear relationship, as the dispersion effect was corrected.

Figure 4 presents the effect of the system parameters on the slope of the calibration curves obtained. As can be seen, the increase of the inserted sample volume (Fig. 4A) and of the residence time in coil C2 (Fig. 4C) allows a higher sensitivity. On the other hand, an increase on the residence time on reactor C2 causes a decrease on observed sensitivity (Fig. 4B). So, the use of a high insertion volume, a short reactor C1 and a long C2 jointly enabled one to obtain the highest slope possible for the calibration curve. Nevertheless, these optimal conditions could not be used for uric acid determinations in urine due to the poor selectivity of the reaction. According to prior studies¹⁴, different species usually contained in biologic fluids (ascorbic and gentisic acids and bilirubine) reduce the iron faster than uric acid. Neither gentisic acid or bilirubine are commonly present in urine, but there are other reducing biological metabolites, or drugs such as ascorbic acid, that are mainly eliminated without being metabolized. Therefore, ascorbic acid was chosen to represent the probable interferences present in urine. It was found that insertion of interferent aqueous solutions promoted the increase of the analytical signal peak height attained at the end of C1 and of C2, being closer to DR when the time delay of the first detection was increased and the sample volume decreased. Using an injection volume of 30 μ l and a 320 cm C1 reactor allowed one to eliminate the interference effect of the studied reductant up to a concentration of 0.23 mmol/l. Under these conditions, the highest intensity of the first analytical signal was recorded at 59 s after insertion of the sample. By selecting a length of 250 cm for C2, a calibration straight line with a slope of 212 mAU l/mmol, was allowed, in the analytical range from 0.01 up to 0.60 mmol/l.

Application to real sample analysis

In the present system, an urine volume (*ca.* 30 μ l) was inserted into a carrier stream (water, 0.63 ml/min),

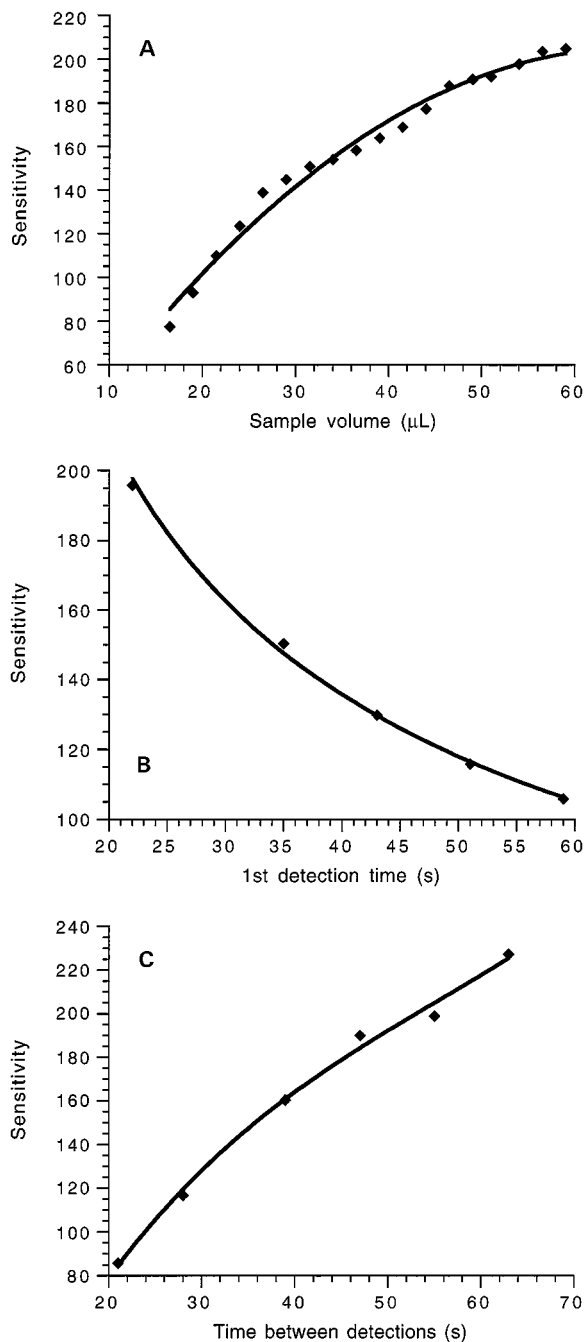


Fig. 4 A) Effect of the sample volume on sensitivity. Sensitivity is defined as $((H2 \times DR) - H1)$. H1 and H2 are expressed in mAU. B) Effect of residence time of the first analytical signal on sensitivity. Sensitivity is defined as $((H2 \times DR) - H1)$. H1 and H2 are expressed in mAU. C) Effect of the time interval between the first and second analytical signal on sensitivity. Sensitivity is defined as $((H2 \times DR) - H1)$. H1 and H2 are expressed in mAU.

by displacement of the mobile slide of the injector/commutator (Fig. 1). Afterwards, the sample plug merged with a reagent channel (1.65 ml/min) and flowed through a coiled reactor (320 cm, 0.8 mm i.d.) towards the detector. After 59 s, the first analytical signal was recorded and at 75 s the injector/commutator was once again displaced leading the detector to its for-

mer position after C2, what allowed one to record the second analytical signal at 106 s.

The accuracy of the proposed system was evaluated by performing determinations of uric acid concentration in 32 urine samples (0.02 to 0.58 mmol/l) and comparing the results with those provided by the conventional procedure. The relationship $C_{\text{FIA}} \text{ (mmol/l)} = 1.019(\pm 0.014)C_{\text{Ref}} \text{ (mmol/l)} - 0.001(\pm 0.009)$; $r=0.997$ (confidence level of 95%) was obtained. C_{FIA} represents the results given by the proposed methodology and C_{Ref} those provided by the conventional methodology.

The precision of the developed method was also evaluated by carrying out 12 replicate injections of two urine samples with uric acid concentrations of 0.24 mmol/l and 0.48 mmol/l. The relative standard deviations assessed for both samples were 2.9 and 2.0%, respectively.

In conclusion, flow injection analysis combined with a multi-site detector is advantageous in low cost automation alternative for kinetic determinations, as shown by this work. Compared with stopped flow systems, the present method does not require any extra device for time control, since the reproducibility of the flow and the length of the tubular paths C1 and C2 ensure the maximum intensity of the analytical signal to be recorded always at the same time (always the same residence time), which contributes to a more reproducible and accurate system. DR was an easy process of overcoming the dispersion effect of single-channel systems with multi-site detection and, therefore, allowed one to use these systems in kinetic determinations. Moreover, the possibility of displacing the detector, and the DR, allow these systems to decrease the interference of the blank signals provided either by the matrix or the interferents present in the samples.

The authors wish to thank Junta Nacional de Investigação Científica e Tecnológica (JNICT) for its financial support through the PBIC/C/QUI/2162/95 project. One of us (J. A. M. C.) would like to acknowledge the PhD grant given by JNICT.

References

1. H. A. Mottola, "Kinetic Aspects of Analytical Chemistry", John Wiley & Sons, New York, 1988.
2. S. R. Crouch, *Anal. Chim. Acta*, **283**, 453 (1993).
3. J. H. Dahl and A. Jensen, *Anal. Chim. Acta*, **105**, 327 (1979).
4. H. Kagenow and A. Jensen, *Anal. Chim. Acta*, **114**, 227 (1980).
5. A. Fernandez, M. A. Gómez-Nieto, M. D. Luque de Castro and M. Valcarcel, *Anal. Chim. Acta*, **165**, 217 (1984).
6. J. Ruzicka, *Anal. Chim. Acta*, **261**, 3 (1992).
7. E. B. Townsend and S. R. Crouch, *Tr. Anal. Chem.*, **11**, 90 (1992).
8. E. A. G. Zagatto, H. Bergamin F°, S. M. B. Brienza, M. A. Z. Arruda, A. R. A. Nogueira and J. L. F. C. Lima, *Anal. Chim. Acta*, **261**, 59 (1992).
9. A. R. A. Nogueira, S. M. B. Brienza, E. A. G. Zagatto, J. L. F. C. Lima and A. N. Araújo, *J. Agric. Food Chem.*, **44**, 165 (1996).
10. A. R. A. Nogueira, S. M. B. Brienza, E. A. G. Zagatto, J. L. F. C. Lima and A. N. Araújo, *Anal. Chim. Acta*, **276**, 121 (1993).
11. A. N. Araújo, J. A. M. Catita, J. L. F. C. Lima and E. A. G. Zagatto, *Fresenius' J. Anal. Chem.*, **360**, 100 (1998).
12. J. A. G. Neto, A. R. A. Nogueira, H. Bergamin F°, E. A. G. Zagatto, J. L. F. C. Lima and M. C. B. S. M. Montenegro, *Anal. Chim. Acta*, **285**, 239 (1994).
13. A. Tabacco, F. Bardelli, F. Meattini and P. Tarli, *Clin. Chim. Acta*, **104**, 405 (1980).
14. F. J. Krug, H. Bergamin F°, E. A. G. Zagatto, *Anal. Chim. Acta*, **117**, 81 (1980).
15. J. D. Artiss, W. M. Entwistle, *Clin. Chim. Acta*, **116**, 301 (1981).
16. J. Ruzicka and E. H. Hansen, "Flow Injection Analysis", 2nd ed., Wiley, New York, 1988.
17. S. Brooks and J. G. Dorsey, *Anal. Chim. Acta*, **229**, 35 (1990).
18. W. E. van der Linden, *Pure Appl. Chem.*, **66**, 2493 (1994).

(Received March 16, 1998)

(Accepted May 21, 1998)