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Stepwise Injection Photometric Determination of Phosphate-Ions in Human Urine

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Abstract

The possibility of stepwise injection determination of analyzed substance in solutions by the standard additions method on example of determination of phosphate-ions in human urine has been shown. The automatic method for determination of phosphate-ions in human urine with the determination range of 2 to 12 mg/l and efficiency of 6 determinations per hour has been developed.

Key words Stepwise injection analysis, standard additions method, phosphate-ions, human urine.

1. Introduction

The automation of chemical analysis methods are important for the clinic labs, which perform the mass-analysis of biological mediums. For the chemical analysis automation the largest spread gained flowing methods such flow injection analysis (FIA) [1] and sequent injection analysis (SIA) [2]. However, these methods are limited for optimization of analytic forms formation of determined substances. This leads to sensibility descent of the flow methods comparing with automated static analogous of these methods. The largest abilities for automation of chemical analysis in terms of securing maximum sensibility the new method of flow analysis has achieved – Stepwise Injection Analysis (SWIA) [3], where conditions of analytical measurements are maximally approached to static ones.

One of important tasks appearing in clinical labs is determination of phosphate-ions in the human urine, necessary for diagnostics for phosphor lack (hypophosphatemia) or excess (hyperphosphatemia) in the metabolism process [4]. FIA [5] and SIA [6] photometric methods of phosphate-ions determination in human urine using the reaction of reduced molibdophosphorous heteropolyacid formation has been developed, the grade graph method was used for analyzed substance determination.

For the elimination of matrix effects influence of urine samples on determination results the stage of discharge extraction of analyzed substance has been included in FIA method [5], when preliminary dilution of urine samples with water is used for SIA method [6], leading to sensibility loss of automated method.

The most proper for photometric analysis of objects with the essential matrix affection as urine is the method of standard additions. The goal of present work was the developing of stepwise injection determination of phosphate-ions in human urine by the method of standard additions.

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2. Experimental

2.1. Reagents

The operational solutions of phosphate-ions have been prepared from 1 g/l solution of phosphate-ions, made by dissolving KH_2PO_4 in distilled water and normalized by indirect chelatometry right before experiment. For this purpose 20 ml of phosphate-ions solution was placed in conic flask, 5 ml of ammonia buffer solution (pH=9.5) and 2-3 drops of 5 g/l ethanol solution of chromogen black T. Solution in flask was titrated by the standard magnesium chloride 0.01 M solution until color change from blue to violet. Determination of exact concentration of 0.01 M magnesium chloride solution was performed by chelatometry in presence of chromogen black T as indicator.

In order to prepare reagents solutions, in accordance with [7] 50 ml of 2.5 M H_2SO_4 solution was mixed, 15 ml of 40 g/l $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ solution, 30 ml of 20 g/l ascorbic acid

solution and 5 ml 3 g/l $K(SbO)C_4H_4O_6 \bullet 0.5H_2O$ solution. Mixed solution of reagents was used to prepare every day.

All used reagents («Reaktiv», Saint-Petersburg, Russia) were qualified no lower than of analytical reagent grade quality.

2.2. Apparatus

SWIA hydraulic scheme for photometric determination of phosphate-ions in human urine was built up on the ground of flow analyzer «PIAKON-30-1» («Rosanalit», Saint-Petersburg, Russia). Photometric detector (λ=670 nm, optical path length of 10 mm), single-channel peristaltic pump, providing stream direction reverse, six-way valve made with polytetrafluoroethylene (PTFE), reaction tube (RT) which is glass tube of 200 mm high and internal diameter of 15 mm, communication pipes of PTFE and internal diameter of 0.5 mm were used in the experiment. For solutions thermostating the reaction tube of SWIA was placed in the special thermostat. Analyzer was managed automatically by computer.

The manifold of SWIA, intended to photometric determination of phosphate-ions by the formation of reduced molybdophosphoric acid reaction is presented on Fig. 1. According to this scheme, in thermostatic reaction tube (4) reverse pump sequentially injects solutions of sample and reagents required. After generation in the reaction tube solution of analytical form colored solution through same channel of reverse pump, used for injection of solutions of sample and reagents, via six-way valve goes to flow detector, where measurement of analytical occurs.

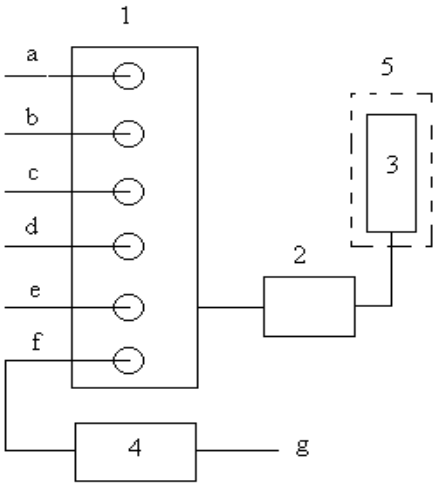

Fig. 1. The manifold of stepwise injection determination of phosphate-ions in urine: 1 – six-way valve; 2 – reverse pump; 3 – reaction tube; 4 – detector; 5 – thermostat; a, b, c, d, e – channels for entering sample, phosphate-ions solution, distilled water, solution of reagents, air, f – channel for entering solutions in detector; g – waste.

2.3. Temperature influence on the analytic form generation

As a preliminary phase influence of temperature in range of 20 to 90 ^{0}C on analytic signal was studied on the standard phosphate-ions water solutions according to scheme presented on Fig. 1. In accordance with SWIA manifold on the initial stage (Fig. 1) in reaction tube (3) with set temperature (from 20 to 90 ^{0}C) by switching tap (1) and reverse pump (2) were entered 2 ml of 10 mg/l phosphate-ions solution (b), 0.5 ml of reagents solution (d) and atmosphere air flow (e), provided solutions mixing in reactionary mix.

Duration of solution mixing in the RT by air flow with the flow rate of 6 ml/min, which is necessary for maximum value of absorbance obtaining, was found equal to 1 min. After that solution from reaction tube by switching of switching valve and reverse of pump was transported from the RT to photometric detector cell (4). The measuring of absorbance (A_n) was carried out in stopped-flow mode, than wasted.

On the second stage washing of communication pipes' with distilled water has been carried out at the same temperature and background signal measurement (A_0) when detector cell was filled up with distilled water.

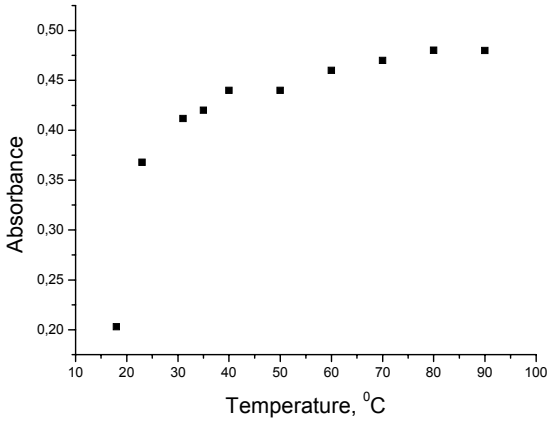

Fig. 2. Dependence of standard solution analytical form absorbance from temperature of photometric reaction time (thermostating time 1 min).

As absorbance value corresponds with the standard solution it was used the difference A_n-A_0. One can see from obtained results (Fig.

2), beginning from temperature of 60 ^{0}C standard solution's absorbance maximum value virtually doesn't change. Therefore 60 ^{0}C temperature was chosen as optimal.

3. Results and discussion

3.1. Formation rate of analytic form in the reaction tube

To determine minimum time for full behavior of reduced heteropolyacid formation reaction in the reaction tube, using in SIA technique (Fig. 1), series of analogous experiments were carried out at different times of solutions thermostating in the reaction tube at fixed temperature of 60 ^{0}C and fixed flow rate of air used for mixing.

Results of investigation of time of analytical reaction behavior in the reaction tube on analytical signal rate are presented at Fig. 3. It was established from the obtained results, the minimum time, required for photometric reaction full behavior in the reaction tube is equal to 60 sec.

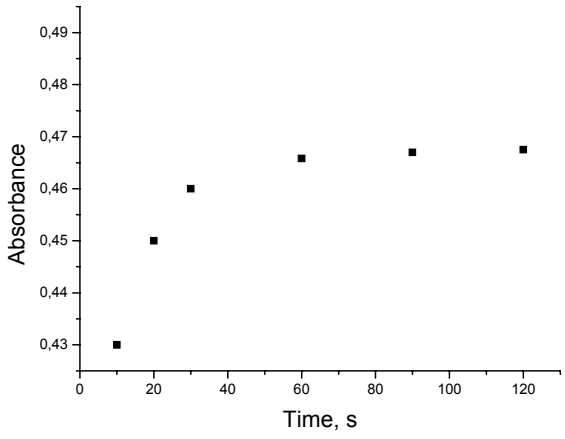

Fig. 3. Dependence of standard solution absorbance from time of photometric reaction carrying out at 60 ^{0}C.

3.2. The methodology of stepwise injection photometric determination of phosphate-ions in human urine.

The optimal conditions of molybdophosphoric acid reduced form formation were used for methodology of phosphate-ions determination developing in human urine by the method of additions.

The variant of two additions has been chosen here, quantity of each addition was varied by changing the ratio of entered phosphate-ions standard solution and distilled water in the RT volumes.

For sample measurement with every addition through switching tap (1) (Fig. 1) by the reverse pump (volume speed of 6 ml/min) (2) in the RT of constant temperature (60 ^{0}C) were sequentially entered certain volumes of urine sample (a), 2 mg/l phosphate-ions solution (b), distilled water (c) and reagents solution (d). The flow of atmosphere air (e) was passed through in course of 60 sec. After that the absorbance value of sample solution with addition (A_x) was measured in the stopped-flow mode, than wasted.

Volumes of samples and solutions added in the RT each time are shown in table 1.

At the final stage washing of communication pipes' with distilled water has been carried out and background signal measurement (A_4), provided entering in detector urine sample, preliminary diluted in reaction tube with distilled water as 1:5.

Table 1. Volumes of samples, entering in the RT and volumes of reagents' solutions for phosphate-ions determination in urine by the standard additions method.

№	Volume (ml)				Comments
	Sample	Phos-phate-ions solution	Distilled water	Reagents' Solution	
1	0.5	0	1.5	0.5	sample
2	0.5	1.5	0.5	0.5	Sample + 1st addition
3	0.5	2.0	0	0.5	Sample + 2nd addition

Shape of obtained analytical signals presents at Fig. 4, which is fixed detector signals, correlate to sample (A_1), sample with additions (A_2, A_3) and background solution (A_4).

Solutions absorbance was calculated as a difference between magnitudes of sample and additions signals and signal of background solution. On the ground of obtained solutions absorbance magnitudes analyzed substance content in sample was found in accordance with formula:

Table 2. Conditions of stepwise injection determination of phosphate-ions in urine.

Time, sec	Tap Position	Pumping direction (-1;0;1)*	Measurement (0;1)**	Comments
5	a	-1	0	Sample goes to RT
20	c	-1	0	Distilled water goes to RT
5	d	-1	0	Mixed solution of reagents goes to RT
60	e	-1	0	Air goes to RT
30	f	1	0	Solution of analytical form goes to detector
15	f	0	1	Stream stops and sample's signal is measuring
10	f	1	0	Waste of the analytical form solution
5	a	-1	0	Sample goes to RT
15	b	-1	0	Standard solution (the 1st addition) goes to RT
5	c	-1	0	Distilled water goes to RT
5	d	-1	0	Mixed solution of reagents goes to RT
60	e	-1	0	Air goes to RT
30	f	1	0	Solution of analytical form goes to detector
15	f	0	1	Stream stops and measuring the signal of sample with the 1st addition is carrying out
10	f	1	0	Waste of the analytical form solution
5	a	-1	0	Sample goes to RT
20	b	-1	0	Standard solution (the 2nd addition) goes to RT
5	d	-1	0	Mixed solution of reagents goes to RT
60	e	-1	0	Air goes to RT
30	f	1	0	Solution of analytical form goes to detector
15	f	0	1	Stream stops and measuring the signal of sample with the 2nd addition is carrying out
10	f	1	0	Waste of the analytical form solution
30	c	-1	0	Distilled water goes to RT
30	f	1	0	Washing liquid waste
5	a	-1	0	Sample goes to RT
25	c	-1	0	Distilled water goes to RT
20	f	1	0	Background solution goes to detector
15	f	1	1	Stream stops and measuring the background signal is carrying out
30	f	1	0	Waste of background solution

* -1 – pump goes clockwise
0 – pump stops
+1 – pump goes counterclockwise
** 0 – no measurement carrying out
1 – registration of detector's signal
Flow speed – 6 ml/min

$$C_x = \frac{(A_3 - A_1) \cdot C_1 \cdot V_2}{(A_2 - A_1)^2 \cdot C_2 \cdot V_3 \cdot 2 \cdot V_1}$$, where C_x – concentration of analyzed substance in sample; A_1, A_2, A_3 – absorbance of sample, sample with first addition and sample with the second addition, consequently; C_1 and C_2 – concentrations of phosphate-ions in the first and the second additions, consequently; V_1, V_2, V_3 – volumes of sample entered in the RT, the first and the second additions, consequently.

To provide the proper order of mixing, it requires quantities of sample and solutions of reagents, sequence and duration of all analyze stages, the matrix was composed, allow to manage analyzer and set the conditions of all the executive parts of apparatus for every moment of time. Every line in this matrix corresponds with the definite stage of analysis, columns answer the state of every executive element. The matrix for phosphate-ions determination in urine is presented in table 2.

4. Conclusion

For inspection of methodology developed samples of urine were analyzed both with developed SWIA method and stationary phosphate-ions photometric methodology determination in water environments, both using the grade graph method [7] with preliminary 10 times dilution of urine samples with distilled water. Comparison of phosphate-ions determination in urine samples, obtained by developed methodology and article [7] (table 3), allows to conclude that results obtained in both methods are virtually identical.

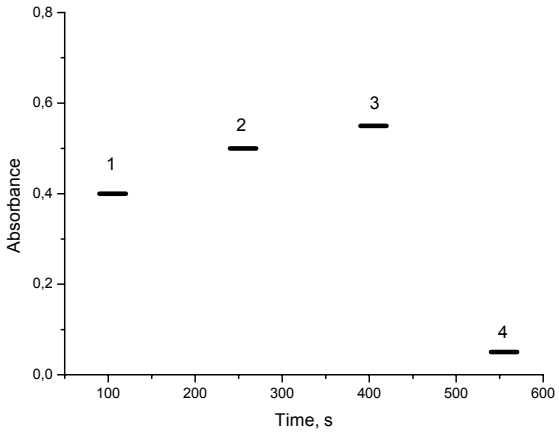

Fig. 4. Analytical signal in SWIA: 1, 2, 3 – sample (11.2 mg/ml), sample with the first addition (1.5 ml 2 mg/l phosphate-ions solution) and sample with the second addition (2 ml 2 mg/l phosphate-ions solution); 4 – background solution.

Table 3. The results of phosphate-ions determination in urine samples (n=5, P=0.95).

Sample	Found, mg/l	
	The developed method	[7]*
1	10.8±0.8	10±1
2	8.4±0.6	8±1
3	11.8±0.8	11±1
4	9.7±0.6	10±1
5	10.5±0.6	10±1
*samples were preliminary diluted 10 times with distilled water		

The developed methodology allows to determine phosphate-ions in urine samples in the range of determined concentrations from 2 to 15 mg/l. Detection limit of 1 μg/l with the volume of sample equal to 0.5 ml and time of one cycle equal to 10 min was achieved.

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