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Determination of antipyrine in saliva using the dispersive liquid–liquid microextraction based on a stepwise injection system

Andrey Bulatov [a], Kseniia Medinskaia [a,*], Darina Aseeva [a], Sergei Garmonov [b], Leonid Moskvin [a]

[a] Department of Analytical Chemistry, Faculty of Chemistry, Saint-Petersburg State University, Saint-Petersburg, pr. Universitetskij 26, 198504 Saint-Petersburg, Russia

[b] Department of Analytical Chemistry, Certification and Quality Management, Kazan National Research Technological University, Kazan 420015, ul. K.Marksa 68, Russia

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ABSTRACT

A fully automated stepwise injection spectrophotometric method for determination of antipyrine in saliva (agent for non-invasive assessment of the activity of the drug metabolizing system in hepatocytes) has been developed. The method is based on the antipyrine derivatization by nitrite-ion dispersive liquid–liquid microextraction (DLLME) of formed 4-nitrosoantipyrine with subsequent UV–vis spectrophotometric detection. Under optimal experimental conditions (0.5 M sulfuric acid, 6×10^{-3} M sodium nitrite, time 6 min) the absorbance of the colored extract at the 345 nm obeys Beer's law in the range of 3–200 μM of antipyrine in saliva. The LOD, calculated from a blank test, based on 3σ, found to be 1 μM. The relative standard deviation for the determination of 50 μM antipyrine was 4.5% ($n=10$). The proposed method was successfully applied to the determination of antipyrine in saliva and the analytical results agreed fairly well with the results obtained by reference HPLC method.

1. Introduction

A wide variety of various foreign or toxic substances (drugs, pesticides, food preservatives, etc.) are metabolized by microsomal oxidase (MO) enzyme system in hepatocytes. This system serves as a route of detoxification and, in contrast, also as a route of metabolic activation to yield reactive metabolites which initiate toxic and carcinogenic events. Moreover they are involved in metabolism of number of endogenous compounds (steroid hormones, prostaglandins, bilirubin, etc.) [1,2].

There are invasive and non-invasive methods for determination of MO activity. Invasive methods include liver biopsy to determine the amount of enzymes in vitro [3–5] but the sample preparation of liver tissues is labor-intensive and complex process and is also painful for patients. The activity of MO system of liver is normally examined by non-invasive measuring the rate of model drug biotransformation that is only metabolized by the hepatic P-450 enzymes [6]. The procedure based on per os ingestion of model drug followed by determination of its pharmacokinetics. This drug should be rapidly and completely absorbed, it should also have no side effects from the used dose and it should not induce or inhibit its own metabolism. As model drug, antipyrine, aminopyrine, theophylline, phenobarbital and others [7–11] are used in clinical practice.

Antipyrine test was found to have extensively wide application because of its low toxicity [12]. Antipyrine is one of the antipyretic and analgesic drug, it is chemically 2,3-dimethyl-1-phenyl-3-pyrazolin-5-one. Antipyrine test is based on the estimation of the pharmacokinetic parameters of antipyrine elimination in biological fluids (saliva, urine or blood) after its oral ingestion. Saliva is used for therapeutic monitoring of a variety of drugs. Moreover, the easy noninvasive, stress-free nature of saliva collection makes it one of the most accessible body fluids to obtain.

Antipyrine has high bioavailability and secreted in saliva with saliva/plasma concentration ratio close to unity [13]. It is negligibly bound with proteins, and its volume of distribution is equal to that of the total-body water, so that antipyrine clearance can be accurately estimated with a single saliva sample.

Several methods have been developed for the determination of antipyrine in biological fluids including capillary electrophoresis [14], electrochemistry [15], radioimmunoassay [16], mass spectrometry [17,18], chromatography [19–21] and spectrophotometry [22,23]. Limit of detection for determination of antipyrine for chromatography, electrophoresis and electrochemistry is 1 μM, but

* Corresponding author. Tel.: +7 911 0010761.
E-mail address: ksmed90@gmail.com (K. Medinskaia).

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proposed techniques have a range of limitations for automation. These techniques involves long stages of sample preparation, using large number of chemicals.

For effective implementation of antipyrine test with high-performance into clinical practice, it would be perspective to use the flow methods. Flow methods are well-established analytical tool for solving problems of routine analysis. Flow methods with spectrophotometric detection can easily and effectively replace complicated and expensive chromatographic separation methods, especially in analysis of biological fluids.

Stepwise injection analysis (SWIA) is the universal solution for the automation of chromogenic reactions in which the equilibration in the reaction is reached and dispersion of the reactants is prevented [24,25]. SWIA manifold is a hybrid analyzer exploiting characteristics of both flow and batch systems. It combines the advantages of automated control of flows such as high throughput, complete and precise control of reactant volumes and timings of operations, low cost, low consumption of the reagents and low waste production with the flexibility and the versatility of mixing chamber (MC).

The purpose of this work was to develop a simple and fully automated non-invasive method for assessment of the activity of MO system, based on the determination of antipyrine in saliva by using SWIA.

2. Experimental

2.1. Reagents and materials

All chemicals were of analytical reagent grade. Ultra-pure water from Millipore Milli-Q RG (Millipore, USA) was used for preparation of solutions and dilution. The stock solution of 500 μM antipyrine was prepared by dissolving of the corresponding weight of antipyrine (Aldrich) in water. The prepared solutions of analyte were standardized by the method that described in [26]. This solution was stored in a dark place at 5 °C and used within 60 days. The working solutions of analyte were daily prepared by appropriate dilution of the stock solution with water. Aqueous solutions of sodium nitrite and sulfuric acid were used for antipyrine derivatization. Pills of pharmaceutical-grade of antipyrine (OJSC "Tatchempharmpreparaty") were used as model drug. Methanol–phosphate buffer solution as mobile phase was prepared by mixing 55% phosphate buffer (pH=7) and 45% of methanol.

2.2. Sampling and sample preparation

A pill containing 600 mg of antipyrine was ingested once per os by healthy patient to estimate the drug metabolizing capacity of liver. After pre-rinsing the oral cavity with water, saliva was collected every three hours during 12 h after antipyrine ingestion in polypropylene conical tube with a volume of 5 mL. 2 mL of collected saliva was put in polypropylene conical tube containing 3 mL of water for centrifugation. The prepared solution was centrifuged for 5 min at 5000 rpm. 1 mL of supernatant was transferred to MC and SWIA was carried out.

2.3. Manifold and apparatus

The SWIA-DLLME manifold (Fig. 1) includes: two solenoid valves (Cole-Parmer, USA), peristaltic pumps MasterFlex L/S (Cole-Parmer, USA) ensuring a reverse flow (flow rate is from 0.5 to 5 mL min^{-1}), multisyringe module 4S (Crison, Austria), mixing chamber (MC) (PTFE 10 mm in i.d., 5 cm at height), MC connected with a source of visible light PX-2 (Ocean Optics, USA) and fiber-optic spectrometer USB 4000 (Ocean Optics, USA) and communication tubes (PTFE, 0.5 mm in i.d.).

HPLC analysis was carried out on a Shimadzu LC-20 Prominence liquid chromatograph (Shimadzu Corporation, Kyoto, Japan) with UV detection (243 nm). The chromatographic separation was achieved by Supelco C18 HPLC column (250 × 4.6 mm, 5 μm particles size) in a gradient elution mode.

2.4. Procedure for the SWIA-DLLME determination of antipyrine

At the first stage of the measurements, the components for antipyrine derivatization were sequentially delivered through the ports of valve (1) by movement of the peristaltic pump (2) into MC (3) in the following order: 1 mL of supernatant (port a), 1 mL of 0.5 M sulfuric acid solution (port b) and 1 mL of 6 mM sodium nitrite solution (port c). To provide the introducing of solutions into the system in reproducible way, the order of mixing and the amount of samples and solutions of reagents, the sequence and duration of all the stages of the analysis, the program which allows to run the analyzer and actuator components state in each time period was made. To stir the reaction mixture, a flow of nitrogen gas was passed through the port d at a rate of 2 mL min^{-1} during 6 min.

At the second stage, the 0.75 mL of extractant mixture (consisted of acetonitrile and dichloromethane, in ratio 3:2) was delivered through the port e of the valve (4) by movement of the syringe pump (5) into MC (3) at a rate of 5 mL min^{-1}. A flow of nitrogen gas was passed through the port d at a rate of 2 mL min^{-1} during 2 min to reach the extraction equilibrium between water and organic phases in MC. The extraction efficiency was 95%.

At the final stage, the absorbance was measured directly in MC under stop-flow conditions (λ=345 nm). The manifold was washed out to eliminate the memory effect. During washing stage all channels and MC was filled with mixture of water and acetonitrile (1:1). The described sequence of the operations was repeated but water was injected instead of saliva sample in order to receive the blank absorbance.

2.5. Procedure for the HPLC determination of antipyrine

The results of SWIA-DLLME determination of antipyrine were compared with those obtained by means of HPLC method [19]. For this purpose, 1 mL of chloroform was added to 1 mL of supernatant and the mixture was mixed properly. At the next stage, the mixture was centrifuged during 2 min at 3000 rpm. After that, organic phase was collected using the syringe and put in polypropylene conical tube. Organic phase was evaporated from tube using water bath under the flow of argon. Then 0.5 mL of water was added to the solid residual, the solution filtered and finally 200 μL of the solution was injected in the loop of chromatograph. Measurements were carried out in the gradient mode, the column temperature was 35 °C. Mobile phase was methanol–phosphate buffer solution.

3. Results and discussion

3.1. Optimization of derivative reaction

Determination of antipyrine is based on the reaction of its derivatization in the presence of nitrite-ions [22]. The reaction proceeds in an acid medium with formation of colored 4-nitrosoantipyrine (λ_{max}=345 nm):