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DETERMINATION OF ISONIAZID IN HUMAN URINE BY SPECTROPHOTOMETRIC METHOD

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ABSTRACT

A simple, rapid, sensitive and accurate spectrophotometric method for the determination of isoniazid in urine has been developed. The method is based on the formation of an orange yellow colour complex between isoniazid and ammonium metavanadate in an acid medium (pH = 1.80 - 2.20). The resulting complex is stable for 12 hours and has an absorption maximum at 420 nm. Beer's law is obeyed in the concentration range of 1.37 - 13.70 µg/ml (R^2 = 0.998) with limit of detection (LOD) being 0.28µg/ml. The molar absorptivities were$1.5.10^4$ $l.mol^{-1}.cm^{-1}$. This method was applied to the determination of isoniazid acetylation rates (phenotypes) in healthy volunteers, patients with hepatitis B and diabetes type 2.

Keywords: N-acetyltransferase 2 (NAT2), acetylation phenotype, isoniazid, spectrophotometry, urine.

1. INTRODUCTION

The acetylation is an important process of conjugate in phase II metabolism of drugs containing amine functional groups, especially aromatic amines as procainamide, hydralazine, dapson, sunfanilamide, sulfasalazine, mesalazine, isoniazid and other drugs. The bimodal distribution is characteristic of this process, and expressesthefast and slow metabolic rates. The difference of the metabolism rate in the acetylation is because of the difference in activity of the enzyme N-acetyltransferase (NAT) and is present in the liver and genetic characteristics of the human race throughout the world [1, 2].

Isoniazid (INH) is one of the main drugs which are effective in theprevention and treatment of tuberculosis (TB). So far, isoniazid and rifampicin, ethambutol, pyrazinamide, streptomycin and thiacetazone remain the essential drugs to treat this disease. After entering the body orally, isoniazid is absorbed rapidly and completely from the gastrointestinal tract. In humans, under the action of the enzyme N-acetyltransferase 2 (NAT2), the main products of isoniazid is metabolizationare acetyl isoniazid and diacetylhydrazine (Figure 1) [3, 4].

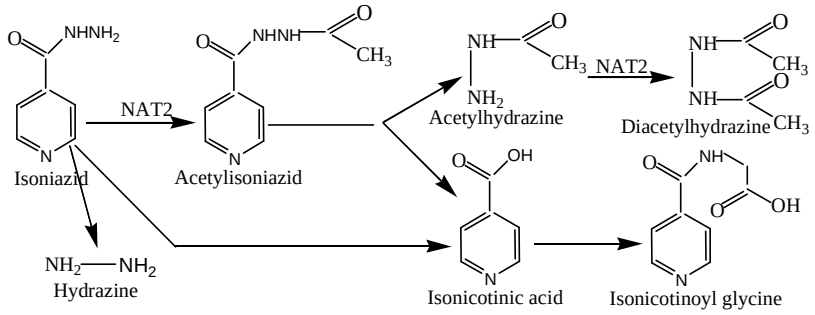

Figure 1. Diagram of isoniazid metabolization in the human body [5].

To determine the N-acetylation phenotype in humans with isoniazid,several authors have evaluated the half-life of isoniazid ($T_{1/2}$), concentration of isoniazid, the ratio of acetyl isoniazid content and isoniazid, "AINH / "INH" in different biological solutions(plasma, saliva) (Table 1).

Table 1. Results of determining the acetylation of isoniazid.

The dose (oral)	Sampling time	Biological solution	Fast acetylation	Slow acetylation	References
450 mg / human body	6 hours after ingestion	Urine	% free INH≤ 7	% free INH > 7	[1]
5 mg/kg human body	6 hours after ingestion	Plasma	$T_{1/2} \leq 110$ min	$T_{1/2} \geq 120$ min	[5]
10 mg/kg human body	12 hours after ingestion	Plasma	$T_{1/2} \leq 110$ min	$T_{1/2} > 110$ min	
10 mg/kg human body	6 hours after ingestion	Plasma	$C_{INH} < 2,5$ μg/ml	$C_{INH} \geq 2,5$ μg/ml	
20 mg/kg human body	3 hours after ingestion	Urine	$\frac{AINH}{INH} \geq 1,18$	$\frac{AINH}{INH} < 0,36$	
5 mg/kg human body	3 hours after ingestion	Plasma	$\frac{AINH}{INH} > 0,7$	$\frac{AINH}{INH} \leq 0,7$	[6]
200 mg/ human body	6 hours after ingestion	Saliva and Plasma	$T_{1/2} < 130$ min	$T_{1/2} > 130$ min	[7]
200 mg/ human body	3 hours after ingestion	Saliva and Plasma	$\frac{AINH}{INH} > 1,5$	$\frac{AINH}{INH} < 1,5$	
10 mg/kg human body	3 hours after ingestion	Plasma	$\frac{AINH}{INH} \geq 0,48$	$\frac{AINH}{INH} < 0,48$	[8]

However, the determination of N-acetylation phenotype according to the above methods encounter some disadvantages. For instance, the process to handle the samples is complicated or it needs accurate kinematic studies of the metabolism of isoniazid. On the other hand, the analysis method requires to have high sensitivity using expensive equipment such as high-

performance liquid chromatography with different detectors (HPLC-UV, PDA, MS, MS / MS). The determination of acetylation based on the percentage of excreted isoniazid in urine [1] by spectrophotometric method has some advantages like simple sample preparation techniques, analysis method of high sensitivity and accuracy.

Determinations of isoniazid medicines and biological solutions by spectrophotometric methodsare based on the use of organic reagents: 4 dimethylaminobenzaldehyde, 7-chloro-4,6 dinitrobenzofuroxan, 2,4,6-Trinitrobenzenesulfonic acid, mixtures of Fe (III) and 1,10 - phenanthroline, a mixture of Cu (II),thiocyanate ion and reagent 4-(2-pyridylazo) resorcinol (PAR), a mixture of Cu (II) and neocuproin ... However, the disadvantages of using these reagentsare that the reaction is often conducted through many phases and lasts a long time[9].

In this paper, we determined the percentage of isoniazid excreted in urine based on complex formation with ammonia metavanadate reagents in an acidic medium by spectrophotometric method. We have applied this method to determine the acetylation phenotype in healthy volunteers as well as patients with type 2 diabetes and hepatitis B.

2. EXPERIMENTAL

2.1. Chemicals and equipment

The chemicals used in the study were very pure for effective analysis. Isoniazid 99% and 99% metavanadate ammonium reagents were purchased from the company Sigma-Aldrich (USA), sulfuric acid, isoniazid products by the pharmaceutical company Semashko (Russia).

Original isoniazid solution (1.37 mg/ml) was prepared by weighing an exact amount of isoniazid and then dissolving it in a carefully measured amount of ethanol. A less concentrated solution was prepared from a stock solution by diluting it with ethanol. 0.2 % ammonium metavanadate reagentin 2 % H2SO4 solution was mixed from equal exactly weight of metavanadatethen dissolving it in 2M H_2SO_4 solution.

All spectrophotometric measurements were performed on the UV-Visible recording spectrophotometer SPECORD® 50 (Germany) using 1 cm quartz cuvettes. Hanna HI 2211 pH meter (Romania) was calibrated daily by standard solutions with pH = 4.00 and pH = 7.00 before measuring.

2.2. Construction of calibration curve

Take 0.3 ml of urine which does not contain isoniazid in the 10 ml volumetricflask, adding standardized isoniazid solution to get concentrations in urine solution of 0; 1.37; 2.74; 5.48; 8.22; 10.96; 13.70 µg /ml. Then add to each volumetricflask 2 ml of 0.2 % ammonium metavanadate in a solution of 2 % H_2SO_4 and norms to the mark with twice distilled water. Comparative solutions were also prepared similarly to the above with no additional conditions for isoniazid standard solutions. The absorbance of the studied solution was then measuredat 420 nm. Calibration was set for each studied subject.

2.3. Preparation of solutions

Each volunteer drank 450 mg isoniazid with 200ml of pure water. Urine samples were collected at times of 0, 2, 4 and 6 hours after taking isoniazid.Research solutions as well as reference solution were prepared in 10 mlvolumetric flasks. Take 0.3 ml of urine from

volunteers in 10 ml flask, add 2 ml into a bottle of 0.2% ammonium metavanadate reagent in 2% H_2SO_4 solution and fill to themark with twice distilled water. Comparative solutions were also prepared in a similar way as the study fluid, addingurine which does not contain isoniazid. Then, the measurement of the optical density at 420 nm of research solutions were compared to therespective solutions.

2.4. Statistical analysis

The limit of detection (LOD) and quantification (LOQ) of the method are given by$3.\frac{SD}{b}$and $10.\frac{SD}{b}$ respectively, relative standard deviation (RSD (%))= $\frac{SD}{\overline{X}}.100.$; where SD is the standard deviation, b is the slope of the calibration curve equation, $\overline{X}$" is the average value of the measurement.Calculation and processing of data were done using the programs Statistica 6 (US) and MS Office 2007 (Excel).

2.5. Volunteers

One hundred and ten healthy volunteers (50 male and 60 female) were chosen for the study. They are all Russian citizens with an average age of 23 years (range 20 - 35 years), the average height of 165 cm (range 153 - 185 cm), and an average weight of 63 kg (range 55 - 92 kg). None of the volunteers has chronic diseases (liver, kidneys, digestive ...), they do not smoke, do not use the drugs, drink neither alcohol nor caffeine containing beverages both before and during the study.

Twenty-four patients with hepatitis B status (14 male and 10 female), average age 37 years (range from 21 - 51 years old), the average height of 173 cm (range 151 - 193 cm), average weight of 75 kg (range 53 – 109 kg).

Sixty-one patients with type 2 diabetes (17 male and 44 female) with disease duration from 1 - 24 years, average age 63 years (range from 47 - 78 years old), the average height of 168 cm (range 150 - 182 cm), the average weight of 67 kg (range 58 – 82 kg).

3. RESULTS AND DISCUSSION

3.1. Electronic absorption spectra of the complex between isoniazid and the ammonium metavanadate reagent

Electronic absorption spectra of ammonium metavanadate reagents, urine (does not contain isoniazid) with ammonium metavanadate reagents and research solution (complex between izoniazid and ammonium metavanadate reagent) at pH = 2.00 is shown in Figure 2. The results show that compared with the electronic absorption spectrum of ammonia metavanadate reagent and the urine (containing noisoniazid) with ammonium metavanadate reagents, the peak of electronic absorption spectrum of research solution shifts to a longer wavelength region and reaches the maximum value at λ_{max}= 420 nm. At this wavelength, the influence of reagents and urinary constituents on electronic absorption spectrum of research solutions is negligible. In the next experiment, we measured absorption of the solution study at the optimal wave length of 420 nm.

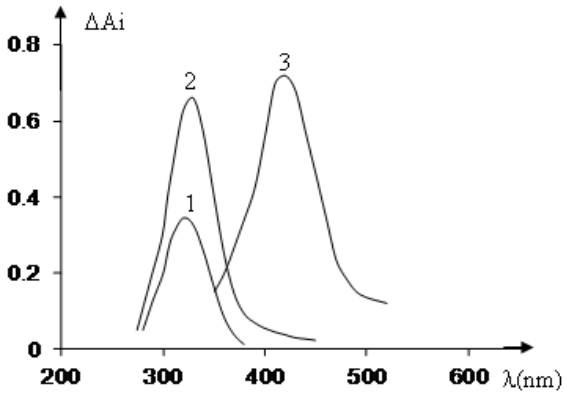

Figure 2. The absorption spectra: 1-Reagent ammonium metavanadate ($1.7.10^{-2}$M, pH = 2.00), 2-Urine (containing no isoniazid) with ammonium metavanadate reagent (4.10^{-3}M, pH = 2.00), 3-Research solution (pH = 2.00).

3.2. Optimal conditions for the complex formation between isoniazid and ammonium metavanadatereagent

The absorption spectra of research solutions at different pH values (Figure 3) show that the electronic absorption spectrum of the complex between isoniazid and ammonium metavanadate reagent is formed at low pH values (1.80 to 2.20) and the maximum absorbance at 420 nm. As pH increased, the optical density of the solution reduced and when the pH = 6.86 research solution gave the absorption intensity shifted to shorter wavelengths. In the next study, we used 2 % H_2SO_4 solution to maintain optimal pH value. Using the method of continuous variations and mol ratio,we defined complex components in a ratio of 1:1, stable complex after 10 minutes preparation and stable complex for 12 hours after preparation.

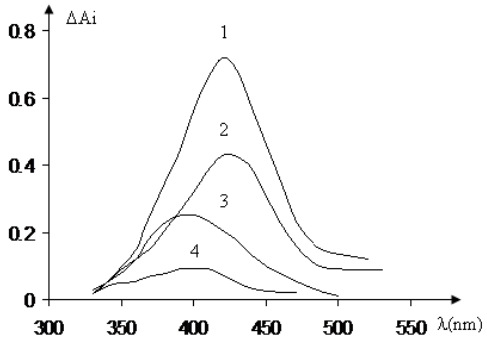

Figure 3. The absorption spectra of complexes between isoniazid (10^{-4}M) and ammonium metavanadate reagent (4.10^{-3}M) at different pH values: 1- pH 2.20; 2- pH = 4.50; 3- pH = 6.86; 4- pH = 7.50.

3.3. Validation of analytical methods

Concentrations obeying Beer's law (μg/ml) range from1.37 to 13.7, the calibration equationis A = 0.0533Cx (μg/ml) + 0.0007. The correlation coefficient R^2 = 0.998, and the relative standard deviation (RSD (%)) = 2.16 %. The molar absorption coefficient of the

complex ε = $1.5.10^4$l.mol^{-1}.cm^{-1}. The limit of detection (LOD) = 0.28 (μg/ml)and the limit of quantitation (LOQ) = 0.94 (μg/ml).

Table 2. Accuracy of quantitative evaluation of isoniazid in the urine samples by spectrophotometric method (n = 4, p = 0.95).

INH taken (μg/ml)	INHfound (μg/ml)	RSD(%)
1.37	1.36 ± 0.02	1.5
2.74	2.75 ± 0.25	1.5
5.48	5.41 ± 0.27	2.4
8,22	8.02 ± 0.25	3.6
10.96	10.83 ± 0.26	1.8

Thus, using spectrophotometric method for determination of isoniazid in the human urine meets the standardization of analytical methods of substances in biological fluids. So, we can use the method developed above to determine acetylation phenotype in human volunteers.

3.4. Applying analytical methods to determine the acetylation phenotype in human volunteers

To classify the volunteers having fast or slow acetylated speed (≤ 7 (> 7) one uses the isoniazid percent excreted in urine after 6 hours [1]. Results ofthe distribution percentage of fast and slow acetylation phenotype in healthy volunteers and patients with type 2 diabetes and hepatitis B are shown in Table 3 and Figure 4.

Table 3. Results of classifying NAT-2 enzyme activity by isoniazid in healthy volunteers, patients with hepatitis B and diabetes type 2 (Mean ± SD).

Acetylation phenotype	NAT activity (Percent excreted of isoniazid,%)		
	Healthy volunteers (n=110)	Patients with hepatitis B (n=24)	Patients with type 2 diabetes (n = 61)
Fast	3.12±0.25	4.48±0.56	4.80±0.80
Probability distributions	p<0.001	p<0.001	p<0.001
Slow	9.40± 1.37	11.35±1.95	13.50±1.50

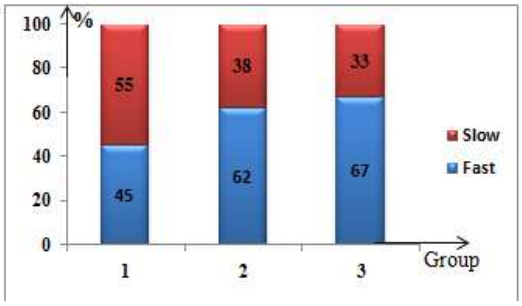

Figure 4. Distribution of acetylation phenotypes: 1 -healthy volunteers (n = 110)
2- type 2 diabetes patients (n = 61), 3- hepatitis B patients (n = 24).

The results in Table 3 and Figure 4 indicate that after 6 hours, the percentage of free isoniazid excreted in the healthy volunteers of fast rate metabolism and slow acetylation are 3.12 ± 0.25 % and 9,40 ± 1.37 % ($p < 0.001$), respectively. Fast and slow acetylation accounted for 45 % (n = 50) and and 55 % (n = 60), respectively. These results are similar to those reported in [1].16 hepatitis B patients showed fast acetylation rate (67 %) and 8 patients showed slow acetylation rate (33 %). Thus, the rapid acetylation in patients with hepatitis B prevailed and increased 22 % compared to healthy volunteers. For patients with type 2 diabetes we observed 38 patients with fast acetylation rate (62 %) and 23 patients with a slow rate (38 %). Thus, similar to patients with hepatitis B, fast acetylation phenotype in patients with type 2 diabetes is also dominant (63 %) and an increase of 18 % compared to healthy volunteers. This can be explained by the reduction in the metabolism of fatty acids synthesis from acetyl coenzyme A (consequences of insulin deficiency).

We ascertained that acetylation speed depends on disease duration for diabetic patients by classifying patients with disease duration of over and under 10 years (Figure 5). For patients with type 2 diabetes with disease duration less than 10 years, we observed no differences between fast and slow acetylation phenotype (53 and 47 respectively %) ($p > 0.05$). However, patients with disease duration of 10 years or more tend to increase faster metabolism of patients (72 %), this can be explained by the metabolic carbohydrates in the body, thereby causing N-acetyltransferase enzyme induction 2 and increases the metabolic rate faster acetyl in these patients.

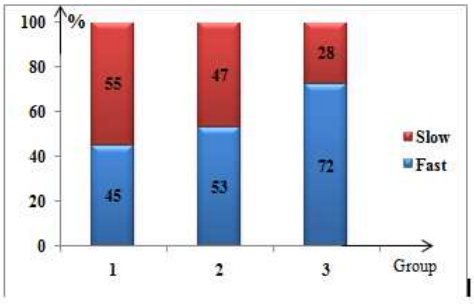

Figure 5. Distribution of acetylation phenotype as dependent on diabetes duration:
1 - healthy volunteers (n = 110), 2- under 10 years, 3 -on 10 years.

4. CONCLUSIONS

Our study shows that the ability to use ammonium metavanadate as reagents to determine the amount of isoniazid excreted in urine has many advantages such as not requiring expensive equipment, and boasting analytical methods which are rapid, selective and relatively sensitive. Therefore, this method may be applied to determine phenotypic acetylation and pharmacokinetic study of isoniazid excreted in human volunteers.

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TÓM TẮT

XÁC ĐỊNH HÀM LƯỢNG ISONIAZID TRONG NƯỚC TIỂU NGƯỜI BẰNG PHƯƠNG PHÁP TRẮC QUANG

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Phương pháp đơn giản, nhanh, nhạy và chính xác đã được xây dựng để xác định isoniazid trong nước tiểu bằng phương pháp trắc quang. Cơ sở của phương pháp dựa trên sự hình thành phức màu vàng cam giữa isoniazid với thuốc thử amoni metavanadat trong môi trường axit (pH = 1,80 - 2,20). Phức hấp thụ cực đại tại 420 nm và bền trong khoảng 12 giờ sau khi chuẩn bị. Khoảng nồng độ tuân theo định luật Bia là 1,37 - 13,7(µg/ml), giới hạn phát hiện (LOD) là 0,28 µg/ml, hệ số hấp thụ mol của phức $\varepsilon = 1{,}5.10^4$ $l.mol^{-1}.cm^{-1}$. Phương pháp đã được áp dụng để xác định kiểu hình acetyl hóa ở người tình nguyện khỏe mạnh, bệnh nhân viêm gan B và tiểu đường loại 2.

Từ khóa: N-acetyltransferase 2 (NAT2), kiểu hình acetyl hóa, isoniazid, trắc quang, nước tiểu.