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Research Article

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Isoniazid Metabolism Monitoring in Libyan patients using HPLC Method

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ABSTRACT

We have tried to assess the acetylation process in tuberculosis a sample of Libyan patient, Simultaneous measurement of isoniazid (INH) and its main acetylated metabolite acetylisoniazid (AcINH) in plasma is realized by high-performance liquid chromatography (HPLC). INH and its main metabolite in human plasma were measured after taken single oral dose (200-300 mg), after 3 hours the samples of patient´s venous blood (4 ml) were collected into heparinized tubes and immediately centrifuged. The plasma samples were then deproteinized by trichloroacetic acid. The analytes separated by HPLC on pinnacle II C18 column (water). Nicotinamide is used as internal standard. The mobile phase is 0.05 M ammonium acetate buffer (pH 6) acetonitrile (99:1, v/v). The detection is by ultraviolet absorbance at 275 nm. Retention time recorded for INH, AcINH and the internal standard were 7.3, 9.9 and 14.2 min respectively. The result obtained showed that the plasma concentration of INH to 48% patient showed slow acetylation, while 52% patients are high acetylator. More than half of TB patients have defect of metabolism isoniazid.

Keywords: Isoniazid, Acetylisoniazid, HPLC.

INTRODUCTION

Tuberculosis (TB) is a common and often deadly infectious disease caused by various strains of mycobacteria, usually *Mycobacterium tuberculosis* in humans which was discovered in 1882 by Robert Coch. Isoniazide with Rifampin are the keystones drug most widely used for treatment such type of infectious disease [1]. Isoniazide is also known as isonicotinylhydrazine (INH), it is an organic compound that is the first-line antituberculosis medication in prevention and treatment . It was first discovered in 1912, and later in 1951 was found to be effective against tuberculosis [2]. The minimal inhibitory concentration (MICs) of isoniazid for wild-type (untreated) strains of *Mycobacterium tuberculosis* is < 0.1 µg. ml^{-1} [3].

Isoniazid is available in tablet, syrup and injectable form (given intramuscularly or intravenously) [4]. Isoniazid is a prodrug and must be activated by a bacterial catalase-peroxidase enzyme called KatG [5]. KatG couples the isonicotinic acyl with NADH to form isonicotinic acyl-NADH complex. This complex binds tightly to the enoyl-acyl carrier protein reductase known as InhA, thereby blocking the natural enoyl-AcpM substrate and the action of fatty acid synthase II system (FAS II). This process inhibits the synthesis of mycolic acid, required for the mycobactrial cell wall [6].

Isoniazid is mainly metabolized to acetylisoniazid (Ac.INH) by *N*-acetyltransferase type 2 in the liver. Ac.INH is devoid of antituberculosis activity. The rate of INH acetylation is controlled by genetic polymorphism, thus all populations studied show a bimodal distribution of the acetylation capacity and individuals can be classified as either slow or fast acetylation [7-9]. Clinical consequences are more important for slow acetylators who are prone to develop major side effects such as peripheral neuropathy and systemic lupus erythematosus, whereas fast acetylators

may undergo therapeutic failure [10]. The therapeutic plasma concentration of INH 1.5 mg/l 3h. after the oral administration to be considered efficient [11].

It appears from these findings that the measurement of INH and Ac.INH plasma concentrations is necessary for the determination of the acetylator phenotype and INH dosing adjustment. This allows to one restore therapeutic efficiency and minimizes the side effect risks. Moreover, measuring Ac.INH plasma levels allows to one ascertain that the most excessive fast acetylator have taken INH medication.

Several methods have been described to measure the INH and its acetylated metabolite (Ac.INH) in plasma, urine and serum. Non chromatographic methods, such as colorimetry [12], spectrophotometry [13] and spectrofluorimetry [14], these methods are time consuming, and tedious addition to lack specificity. For advanced quantitative analysis a number of chromatographic methods have been proposed to allow measurement of INH and Ac.INH in biological fluids such as Gas chromatography method [15], (HPLC) methods using ultraviolet or fluorimetric detection [16-18]. In this study we describe a HPLC method for the simultaneous measurement of INH and its acetylated metabolite (Ac.INH) in plasma based on trichloroacetic deproteinization samples obtained from Libyan TB patients.

EXPERIMETAL SECTION

Chemical and reagents: Isoniazid and its main acetylated metabolite (Ac.INH) were provided by F. Hofman-La Roche (Bale, Switzerland). Acetonitrile, used was of HPLC grade obtained from Merck, Nicotinamide used as chromatographic internal standard (I.S), was obtained from Merck (Darmstadt, Germany). Human plasma was provided by Abe Setta Hospital for Respiratory Disease. Methanol and water were LiChrosolv and provided along with trichloroacetic acid, by Merck. The chemicals, ammonia and acetic acid were of analytical grade.

Apparatus: HPLC system was composed of a pump Model L-306, a variable-wavelength UV_Vis spectrophotometric detector Model L-152, a chromatointegrator Model D-2500 and a Rheodyne loop autoinjector (20 µl), all from Gilson. The column was a pinnacle II C18 5µm (150x 4.6 mm) from waters (Reversed phase).

Chromatographic conditions: The isocratic mobile phase was a mixture of ammonium acetate buffer (0.05 *M*, pH 6)-water (99:1, v/v). The buffers consisted of 0.05 and 0.5 M acetic acid in water adjusted to pH 6 and 8.2, respectively, using ammonium acetate. Stock solutions of analytes were prepared a concentration of 1g/l in methanol and stored at + 4^{o}C. The working solution contains 100 µg/ml of INH and 200 µg /ml of Ac.INH. From the last solution five calibration standards of both INH and Ac.INH were prepared in water or in human plasma using a serial dilution with geometric of 2(INH: 0.5-1-2-4 and 8 µ g/ml; Ac.INH:1-2-4-8 and 16 µ g/ml).

The elution was done at room temperature with a flow-rate of 1.2 ml/min (pressure =90 bar). The analysis was performed at 275 nm with a range of 0.02 AUFS.

Sample processing: The 50 tuberculosis patients were subjected to the experiment; there were 35 males and 15 females. The age of the patients ranges from 16 to 60 years, After fasting overnight, the subjects received a single oral dose of isoniazid (200 mg) INH (*D* =5.2 ±0.3mg/kg body mass). The blood samples (4 ml) were withdrawn into heparinised tubes after 3 hours, centrifuged, and stored at -70 °C until assayed to prevent breakdown of INH or Ac.INH.

To 300 µl samples of plasma or aqueous calibration solutions, we added 250 µl of deproteinization solution which consisted of 15 µg/ml nicotinamide in 10% trichloroacetic acid. The mixture was vigorously vortex-mixed for 1 min and then centrifuged for 10 min at 3000 g. The trichloroacetic supernatant was withdrawn and then was half diluted with the ammonium acetate buffer (0.5 *M*, pH 8.20) in order to neutralize the excess trichloroacetic acid.

The samples then were analyzed by high performance liquid chromatography (HPLC) for the simultaneous determination of plasma concentration of INH and its acetylated metabolite.

RESULTS

Blood sample taken from tuberculosis patient are analyzed by HPLC as mentioned before. The elution result for INH, AcINH and the internal standard were 7.3, 9.9 and 14.2 min respectively. The resulted of simultaneous determination of plasma concentration of INH and its acetaylated metabolite Ac.INH in 50 tuberculosis patient were reported 3hrs. post INH 200 mg oral dose. The metabolic ratio (MR) was calculated for each sample [Table 1]. (MR) obtained by divided AcINH over INH, which widely used for determination of acetylator phenotype (17). Out of 50 subjected studied, 24 (48%) were showed slow acetylation while another 26 (52%) were fast acetylator.

Table.1 INH dose and plasma concentration of INH & Ac.INH at 3 hours after administration to 50 tuberculosis patients

Patient NO.	INH dose (mg/kg of body mass)	INH. Conc. (μg/ml)	Ac.INH Conc. (μg/ml)	metabolic ratio (Rm)
1	4.23	0.56	4.17	7.44
2	3.75	0.70	3.20	4.57
3	3.96	0.56	3.76	6.71
4	4.35	0.42	4.87	11.59
5	5.05	1.11	3.20	2.88
6	5.09	0.70	4.45	6.35
7	4.55	0.83	4.59	5.53
8	5.00	0.56	4.76	8.50
9	4.82	0.83	4.31	5.19
10	4.24	0.70	3.20	4.57
11	4.67	0.83	3.89	4.68
12	4.99	1.25	4.73	3.78
13	4.41	0.70	4.31	6.15
14	5.14	0.70	3.60	5.14
15	5.00	0.50	3.10	6.20
16	4.75	0.56	4.03	7.19
17	3.84	0.70	4.87	6.95
18	5.20	0.97	4.73	4.87
19	4.81	1.81	3.75	2.07
20	5.00	1.00	4.73	4.43
21	5.00	1.25	4.45	3.56
22	5.22	1.25	4.31	3.44
23	5.00	1.81	3.48	1.92
24	4.76	1.53	5.00	3.26
25	5.16	1.66	3.20	1.92
26	4.20	1.25	4.45	3.56
27	4.35	1.11	4.03	3.63
28	4.83	1.25	4.45	3.56
29	5.26	0.90	4.86	5.40
30	4.91	1.81	4.59	2.53
31	4.22	1.25	4.45	3.56
32	5.21	1.11	4.86	4.37
33	4.69	0.41	4.03	4.82
34	4.35	0.19	4.03	21.2
35	4.48	1.39	4.47	3.21
36	5.25	1.39	4.73	3.40
37	4.45	1.53	5.00	3.26
38	4.95	0.97	4.17	4.24
39	5.21	1.66	4.45	2.78
40	5.36	1.11	4.45	4.00
41	4.56	1.53	4.03	2.63
42	5.00	1.11	4.73	4.26
43	4.52	3.06	4.28	1.34
44	5.45	1.11	3.76	3.38
45	5.17	2.36	4.87	2.06
46	5.17	0.42	4.59	10.92
47	4.41	0.28	4.59	16.39
48	4.99	0.42	2.37	5.64
49	4.84	1.67	3.89	2.32
50	5.36	0.417	4.87	11.67

DISCUSSION

HPLC method reported to be suited for identification of INH and Ac.INH. The biological samples were freed from protein, before the method of HPLC applied. Methods using liquid-liquid extraction [19], solid phase extraction [20], or using centrifugal filtering device [16], but all were laborious, time consuming and low sensitivity. In this work a mixture of nictinamide with trichloroacetic acid was used for deprotenize the sample, our results were the same as obtained by using amixture of trichloroacetic acid coupling with *trans*- cinnamaldehyde [11].

To obtained separated INH and AC.INH from the sample, reversed phase HPLC column, type pinnacle II C18 from waters was used, however different HPLC column were used by other worker and all were clamed to be rapid and produced convenient separation [19, 20].

Several publications have been reported that the frequency of acetylator phenotype among the population were different related to different in geographic zone and varies from race to race [21]. Example of these report showed that the slow acetylator phenotypes was predominant as 94% in Saudi Arabian [22], 83% in Egyptian [23], and 61.8% in Moroccan [24]. In contrast slow acetylator frequency distribution show only, 14.6% in Indian [25], 13.1% in Japanese [26], and goes as lower as 5% in Alaskan Eskimos [27] while an equal acetylators distribution between slow and fast were reported in both French and Germany [28,29].

In early study for Libyan population, the frequency disruption of slow acetylation found to be 65% using sulphadiamin as a test drug [30]. This work tried to asses acetylation status in Libyan patient using INH, and resulted shown only 48 % to be slow acetylation.

CONCLUSION

The high-performance liquid chromatography (HPLC) method proposed here proved to be convenient for the simultaneous measurement of INH and its acetylated metabolite Ac.INH. The deproteinization with trichloroacetic acid gives an easy, rapid and convenient separation of analytes from plasma sample. The therapeutic plasma concentration of INH should range from 1-2µg/ml. The results its 48% of patients showed slow acetylation while another 52% of patients have fast acetylation.

More than half or 52% TB patients in Abosetta university hospital for respiratory disease, Tripoli, Libya have defect of metabolism isoniazid.

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