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Journal of Chromatography B, 677 (1996) 339–343

JOURNAL OF CHROMATOGRAPHY B: BIOMEDICAL APPLICATIONS

Determination of isonicotinic acid in the presence of isoniazid and acetylisoniazid
Studies on isonicotinic acid formation from isoniazid in isolated rat hepatocytes

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Received 7 June 1995; revised 3 October 1995; accepted 17 October 1995

Abstract

In comparison with the hepatocytes obtained from intact rats and rats pretreated with phenobarbital or 3-methylchoranthrene, the amount of isonicotinic acid (INA) formed from isoniazid (INH) increased substantially after incubation at 37°C using the pretreated hepatocytes. This suggests an oxidative pathway for INA formation from INH, apart from hydrolysis. In order to explore the exact mechanism of INA formation in the hepatocytes, an HPLC assay for INA in the presence of INH and acetylisoniazid was developed. In this assay, INA was extracted after the preparation of an ion pair with tetra-*n*-butylammonium hydroxide, and analysed using an ODS column and a mobile phase consisting of 0.067 *M* potassium dihydrogenphosphate solution–methanol (96:4, v/v). The method is simple, accurate and especially suitable for INA determination after incubation of INH in isolated rat hepatocytes.

Keywords: Isonicotinic acid; Isoniazid; Acetylisoniazid

1. Introduction

We have previously studied isoniazid (INH), an effective tuberculostatic still employed as a first-line drug, and have made many observations on INH and its metabolites determined by GC–MS [1–5]. However, isonicotinic acid (INA) could not be determined by GC–MS in the presence of INH and its metabolites. HPLC is a good method for the determination of non-volatile and hydrophilic carboxylic acids such as INA without derivatization. Using HPLC, INA formed from INH in isolated rat hepatocytes was separately determined from INH and AcINH; the amount of INA was increased substantially using the hepatocytes after pretreatment with

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SSDI 0378-4347(95)00460-2

phenobarbital (PB) or 3-methylchoranthrene (3-MC) [6]. This suggests an oxidative formation of INA from INH catalysed by cytochrome P450 in the metabolic pathway. In order to elucidate the mechanism in detail using isolated rat hepatocytes, a simplified method for INA determination in the presence of INH and acetylated INH (AcINH) was developed.

There have been many reports on the determination of INH and its metabolites using HPLC, but only a few on the detection of INA. Kubo et al. [7] and Mawatari et al. [8] reported the determination of INA simultaneously with INH and its metabolites in human urine by HPLC. However, those methods were developed for acetyl phenotyping using INH, and determination using fluorimetric detection after a photochemical reaction in a postcolumn reactor are not simple. Hence a simplified method is necessary for the metabolic study on INA formation from INH in the hepatocytes, and a suitable HPLC method for INA determination in the presence of INH and AcINH, involving UV detection without derivatization, was developed.

2. Experimental

2.1. Chemicals

INA and INH were purchased from Nacalai Tesque (Kyoto, Japan), 6-methylnicotinic acid (6-MNA) (internal standard) from Aldrich (Milwaukee, WI, USA), 3-MC from Sigma (St. Louis, MO, USA), sodium salt of PB from Tokyo Kasei Kogyo (Tokyo, Japan) and an aqueous solution of tetra-*n*-butylammonium hydroxide (0.5 mol/l) from Wako (Osaka, Japan). AcINH was synthesized in our laboratory. The purities of INA, INH, AcINH and 6-MNA were checked by the elemental analyses and mass and NMR spectrometry.

2.2. Apparatus and HPLC conditions

The HPLC system consisted of a Shimadzu LC-9A chromatograph equipped with a Shimadzu SPD-10A UV detector. A reversed-phase C_{18} analytical column (TSK-gel ODS 80TM, 250 mm × 4.6 mm I.D., particle size 5 μm) (Tosoh, Tokyo, Japan) was used at 37°C. A mixture of 0.067 *M* potassium dihydrogenphosphate and methanol (96:4, v/v) was employed as the mobile phase. The flow rate was 0.8 ml/min and the UV detector was set at 265 nm.

2.3. Sample preparation

To 1 ml of phosphate buffer solution or hepatocyte solution containing INA, INH and AcINH (pH 7.4), were added 3 ml of ethyl acetate–*n*-butanol (2:1, v/v), 0.3 ml of tetra-*n*-butylammonium hydroxide solution (0.5 mol/l) and 0.1 ml of an aqueous solution of 6-MNA (80 μg/ml). After the mixture had been shaken for 20 min followed by centrifugation for 15 min at 850 *g*, 2.5 ml of the upper organic layer were transferred into a tube which contained 1.0 ml of 0.2% hydrobromic acid. After shaking and centrifugation as above, the upper organic layer was removed by suction with a vacuum aspirator. The remaining lower aqueous solution was frozen at −20°C until analysis and 5 μl were injected into the chromatograph. The experimental conditions for the preparation of isolated rat hepatocytes and drug metabolic studies in the hepatocyte system were the same as described previously [6,9].

3. Results and discussion

3.1. Retention time and selectivity

Fig. 1 shows typical chromatograms of (A) the extract from the hepatocyte solution and (B) the extract from the real sample using INH (0.5 m*M*) as a substrate after the incubation at 37°C for 60 min in the hepatocytes prepared from the intact rats. The retention times for INA, INH and AcINH were 5.46, 11.42 and 10.24 min, respectively, and the detected amounts calculated from the calibration graphs were 60, 270 and 240 μ*M*, respectively, as shown in Fig. 1. No peaks that interfered with INA, INH, AcINH and 6-MNA were detected.

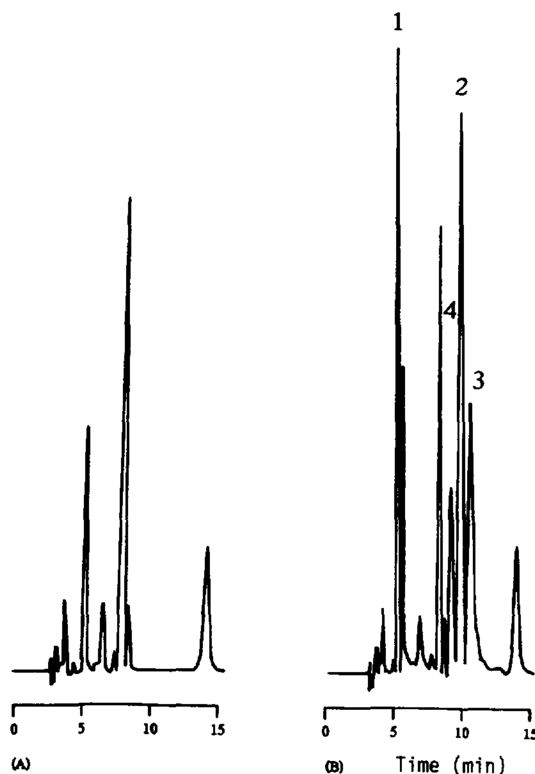

Fig. 1. Chromatograms of extracts from real samples: (A) inactivated hepatocytes and (B) hepatocytes incubated with INH (0.5 m*M*, 37°C, 60 min). Peaks: 1 = INA (60 μM); 2 = AcINH (190 μM); 3 = INH (270 μM); 4 = 6-MNA (I.S.).

3.2. Extraction procedure

The yield of the extraction procedure was investigated by multiple measurements at low, medium and high concentrations. In this assay, the sample was prepared by ion-pair extractions, i.e., (1) extraction of the ion-paired sample into an organic layer and (2) extraction of the ion-pair-broken sample into hydrobromic acid solution. An acidic solution was employed for HPLC. The extraction recoveries of INA, INH and AcINH at all three concentration levels ($n = 6$) were ca. 80%, as shown in Table 1.

3.3. Linearity and limit of quantification

The peak-area ratios of INA, INH and AcINH to the internal standard varied linearly with concentration over the ranges used, i.e., 2–10 μg/ml for INA and AcINH and 5–25 μg/ml for INH, in 0.067 *M* phosphate buffer solution (pH 7.4). For each point of the calibration standards, concentrations were recalculated from the equation of the linear regression curves ($n = 3$, experimental concentrations), i.e., $y = 0.00421x - 0.00196$ ($r = 0.999$) for INA, $y = 0.00637x + 0.00224$ ($r = 1.000$) for INH and $y = 0.00823x - 0.00015$ ($r = 0.999$) for AcINH. Although each concentration of INA, INH or AcINH can be detected even at nanogram levels, the limit of quantification for each compound in the hepatic system was adjusted to be over 2 μg/ml.

3.4. Precision and accuracy

The intra-day precision and accuracy of the assay were tested by multiple measurements of three different concentration levels against the inactivated hepatocytes. The results are summarized in Table 2. The intra-day reproducibility was determined for calibration graphs prepared on the same day in replicate with the same stock solutions according to the proposed method. In spite of sufficient accuracy of the assay, however, each calibration graph should be prepared using the inactivated hepatocytes just before the determination. In this method, to examine the day-to-day reproducibility is meaningless, since hydrolysis of INH and AcINH took place partly in an aqueous stock solution even at −20°C 24 h after the sample preparation. The simultaneous determination of INA, INH and AcINH was performed by HPLC within 24 h in a short time, and then results given in Tables 1 and 2 were obtained.

3.5. INA formation from INH in isolated rat hepatocytes

As shown in Fig. 2, an excellent mass balance chart of INH metabolism was obtained by the method, which is simple, accurate and selective for the simultaneous determination of INA in the presence of INH and AcINH. In the hepatocyte system, INA was a final metabolite of INH and AcINH based on the pyridine ring. Accord-

Table 1
Recoveries of INA, INH and AcINH extracted from spiked samples

Parameter	INA					
	5 μg/ml		20 μg/ml		40 μg/ml	
	100%	Extracted	100%	Extracted	100%	Extracted
Mean ($n=6$)	0.378	0.325	1.451	1.278	2.892	2.283
S.D.	0.011	0.011	0.031	0.021	0.059	0.077
Recovery (%)		85.86		88.05		78.94
	INH					
	10 μg/ml		40 μg/ml		80 μg/ml	
	100%	Extracted	100%	Extracted	100%	Extracted
Mean ($n=6$)	0.728	0.646	2.882	2.445	5.687	4.686
S.D.	0.032	0.050	0.128	0.081	0.232	0.147
Recovery (%)		88.72		84.81		82.40
	AcINH					
	5 μg/ml		30 μg/ml		60 μg/ml	
	100%	Extracted	100%	Extracted	100%	Extracted
Mean ($n=6$)	0.294	0.249	1.603	1.280	3.112	2.590
S.D.	0.011	0.022	0.024	0.048	0.031	0.059
Recovery (%)		84.64		79.87		83.28

Mean values are expressed at the peak-area ratio to 6-MNA (I.S.).

Table 2
Intra-day precision and accuracy of INA, INH and AcINH determination

Concentration added (μg/ml)	Concentration found (mean, $n=6$) (μg/ml)	R.S.D. (%)	Accuracy (%)
INA			
5	4.9	4.2	2.0
20	21.6	1.9	8.0
40	39.2	3.7	2.0
INH			
10	9.6	9.6	4.0
40	40.8	3.7	2.0
80	79.7	3.5	0.4
AcINH			
5	5.5	10.6	10.0
30	29.6	4.1	1.3
60	60.1	2.5	1.7

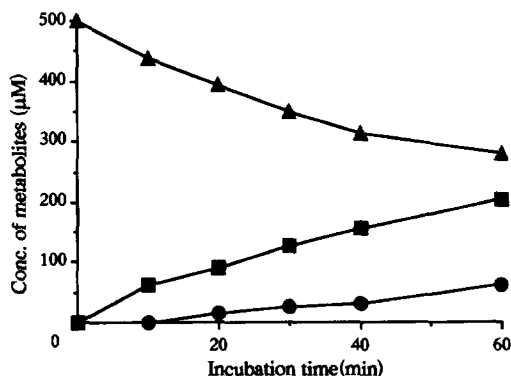

Fig. 2. Formation of INA and AcINH from INH (0.5 m*M*) in isolated rat hepatocytes ($16 \cdot 10^6$ cells/ml, 37°C). ● = INA; ▲ = INH; ■ = AcINH.

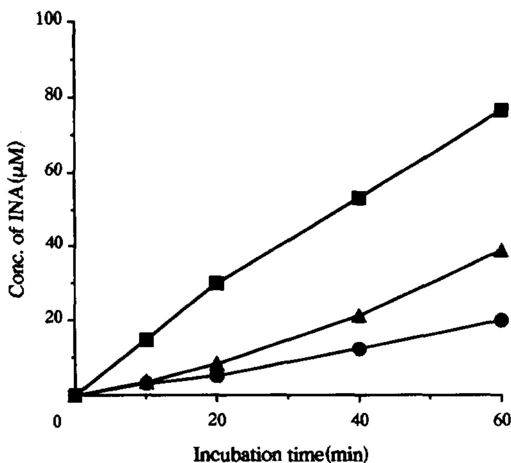

Fig. 3. Effects of PB and 3-MC pretreatment on INA formation from INH (0.5 m*M*) in isolated rat hepatocytes ($8 \cdot 10^6$ cells/ml, 37°C). ● = Intact; ▲ = PB pretreated; ■ = 3-MC pretreated.

ing to the time course of INA formation from INH (0.5 m*M*) shown in Fig. 3, increasing effects of PB and 3-MC pretreatment on INA formation were observed when the experiments were performed under the same conditions as presented earlier [6,9]. The oxidative metabolism of INA formation from INH will be reported separately.

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