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Note

High-performance liquid chromatographic analysis of isoniazid and acetylisoniazid in biological fluids

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Plasma concentrations of isoniazid and acetylisoniazid may be valuable in monitoring compliance with an efficacy of therapy in tuberculosis. They have also been used to measure the acetylator status of patients in order to assess relative risk of development of adverse effects to several drugs.

Several methods are available for the measurement of isoniazid in biological fluids. The most commonly used methods are listed in Table I but each have disadvantages in terms of sensitivity, specificity or time of analysis. Two selective, although relatively insensitive high-performance liquid chromatographic (HPLC) methods have been described [1, 2]. We therefore describe an HPLC method which is ten-fold more sensitive and which has provided superior resolution of isoniazid and acetylisoniazid in our hands.

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TABLE I

SUMMARY OF METHODS AVAILABLE

Type of assay	Biological specimen*	Specificity	Estimated sensitivity (μg/ml)
Colorimetric [5]	S U	Isoniazid	>5
Microbiological [7]	S U	Isoniazid and metabolites	<0.1
Fluorimetric [6]	S U	Isoniazid	0.01
Gas chromatographic [8]	U	Isoniazid and metabolites	<0.5
Gas chromatographic—mass spectrometric [3]	P	Isoniazid and metabolites	0.01
Radioimmunological [9]	S	Isoniazid	<0.05
Liquid chromatographic [1]	P U	Isoniazid and acetylisoniazid	<0.2
Liquid chromatographic [2]	S	Isoniazid and acetylisoniazid	0.3
HPLC (present method)	B S P U	Isoniazid and acetylisoniazid	0.02

*B = blood, S = serum, P = plasma, U = urine.

EXPERIMENTAL

Reagents

Isoniazid and iproniazid phosphate were supplied by Aldrich (Gillingham, U.K.). Acetyl isoniazid was prepared using a modification [3] of the method of Fix and Gibas [4]. The acetonitrile was of HPLC quality and was supplied by Fisons (Loughborough, U.K.). All other solvents and reagents were of analytical quality.

Sample preparation

A schematic representation of the procedure for the analysis of isoniazid and acetylisoniazid is shown in Fig. 1. Heparinised plasma (1 ml), heparinised blood (1 ml) or a 1-ml dilution of urine (1:10, v/v) was placed in a PTFE-lined screw-capped culture tube, and 50 μl of the internal standard solution (containing 2 μg iproniazid), 500 μl phosphate buffer (0.5 *M*, pH 7.4) saturated with sodium chloride, and 10 ml chloroform—butane-1-ol (70:30) were added. The samples were extracted by gentle mixing for 10 min followed by filtration through Whatman IpS phase separating paper. The organic phase was back extracted with 500 μl of 0.05 *M* phosphoric acid by gentle mixing for 10 min. After brief centrifugation, 20 μl of the aqueous phase were applied to the chromatograph.

Chromatography

The HPLC system used was supplied by Laboratory Data Control (Stone, U.K.). The system comprised a Constametric III high-pressure solvent delivery system equipped with a Model 7125 Rheodyne valve and fitted with a Spherisorb nitrile column (250 × 4.5 mm I.D.; particle size 5 μm). The absorbance was measured at 266 nm, at 0.01 a.u.f.s. deflection using a Spectromonitor III variable-wavelength detector. The mobile phase used was 0.01 *M* phosphoric acid in acetonitrile—water (20:80) at a flow-rate of 2 ml/min.

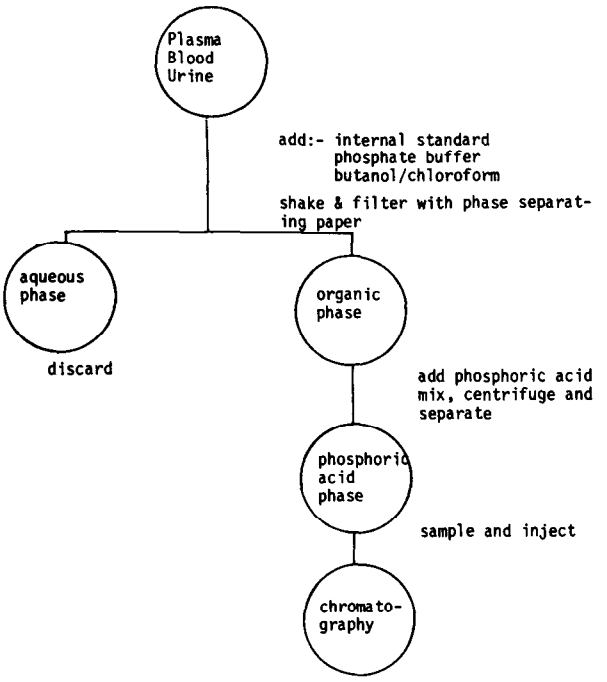

Fig. 1. Flow diagram of the sample preparation for the HPLC analysis of isoniazid and acetylisoniazid.

Chromatograms were recorded and the peak heights integrated using a Chromatography Control Module equipped with a printer-plotter.

Calibration and accuracy

Calibration curves were constructed by adding known masses of internal standard and isoniazid to plasma, blood or urine, and plotting the peak height ratios of isoniazid to internal standard against the amount of isoniazid added. Peak height ratios were used to calculate the amount of isoniazid in unknown samples, and the standard deviation of the normalized peak height ratios was used to determine the accuracy of the method over the range of isoniazid standards employed. The precision of the method was also studied by submitting four replicate plasma samples containing 1 and 10 μg/ml isoniazid to the entire procedure. The same approach was used to calibrate and determine the precision of acetylisoniazid estimations.

To estimate the recoveries for the method, the peak heights of analysed 1-ml samples containing known amounts of isoniazid, acetylisoniazid, and the internal standard were compared to the respective peak heights obtained by injecting equal amounts directly into the chromatograph.

RESULTS AND DISCUSSION

Isoniazid and acetylisoniazid were efficiently extracted from plasma, urine and blood. Salting out of the drug using sodium chloride improved extraction efficiency, whereas ammonium chloride which was previously used by

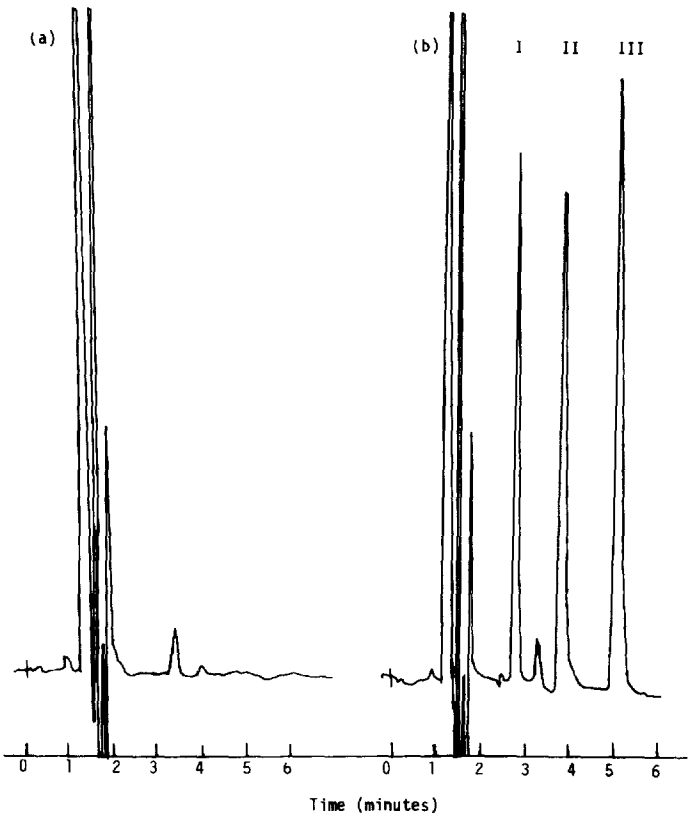

Fig. 2. Chromatograms of (a) control plasma and (b) plasma containing known amounts of (I) acetylisoniazid, (II) isoniazid and (III) the internal standard (iproniazid).

TABLE II

ACCURACY, RECOVERY AND PRECISION OF THE METHOD FOR DETERMINING ISONIAZID AND ACETYLISONIAZID IN BIOLOGICAL FLUIDS

Drug	Biological fluid	Concentration range (μg/ml)	Coefficient of variation of normalized peak height ratios (%)	Recovery (%)
Accuracy and recovery				
Isoniazid	Plasma	0.1—15	4.6	71
Isoniazid	Blood	0.2—15	5.2	68
Isoniazid	Urine	2 —100	3.7	77
Acetylisoniazid	Plasma	0.2—15	3.7	75
Acetylisoniazid	Blood	0.2—15	8.3	72
Acetylisoniazid	Urine	2 —100	4.1	79
Precision (n = 4)				
Isoniazid	Plasma	10	0.9	
Isoniazid	Plasma	1	1.8	
Acetylisoniazid	Plasma	10	4.0	
Acetylisoniazid	Plasma	1	4.1	

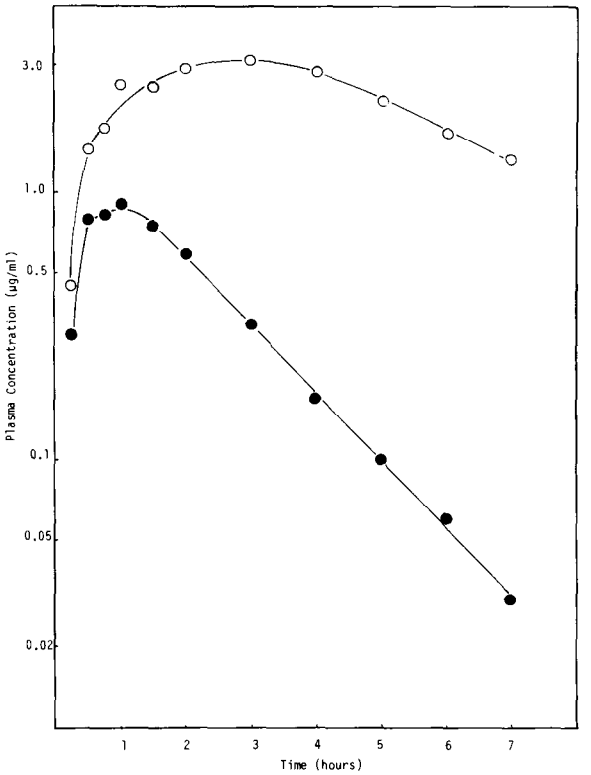

Fig. 3. Semi-logarithmic plot of the plasma concentrations of isoniazid (•) and acetylisoniazid (○) in a healthy male subject for 7 h after a single oral dose of 200 mg of isoniazid.

other authors [1, 5] was found to be much less effective and interfered with subsequent chromatography.

Isoniazid, acetylisoniazid and the internal standard (iproniazid) were well separated by the method described, as were two peaks associated with extraction from plasma (Fig. 2). Retention time for the longest retained compound (iproniazid) was only 6.5 min. The method was accurate and reproducible (see Table II) and could detect as little as 0.02 μg of isoniazid or acetylisoniazid per ml plasma.

The method gave satisfactory results when used to determine kinetic parameters after oral administration of only 200 mg isoniazid to a normal individual (Fig. 3). None of the drugs (ethambutol, streptomycin, rifampicin, pyrazinamide or pyridoxine) often given to patients receiving isoniazid therapy interfered with the estimation of isoniazid or acetylisoniazid. Because of adequate sensitivity of the method the oral isoniazid dose could be reduced by 70% for determining the pharmacokinetic parameters, thus reducing the nausea associated with larger doses. Also only 1 ml of plasma was required for the estimation rather than 3 or 4 ml [1, 5].

Many of the methods described in Table I measure only isoniazid and do not measure its acetylated metabolite [1, 5, 6, 7]. The microbiological method although sensitive, also measures metabolites with anti-tuberculous activity

and determinations take approximately 10 days [7]. The liquid chromatographic method of Saxena et al. [1] can accurately measure acetylisoniazid as well as the parent compound but it is relatively insensitive and uses the method of difference after spiking the samples with additional amounts of drug and metabolite. A recent hydrophilic ion-pair reversed-phase HPLC method is even less sensitive, although faster than the method of Saxena et al. (see Table I). A major disadvantage is a large lump due to serum components which occurs 20 min after injection and could thus interfere with subsequent chromatograms [2].

The gas chromatographic method of Nota et al. [8] does measure acetylisoniazid as well as parent compound but has poor sensitivity. The gas chromatographic—mass spectrometric method [3], although sensitive, requires very expensive equipment.

We have found that the method we describe can be performed on conventional HPLC equipment and up to 40 samples can be processed in a working day.

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