Rapid, specific and sensitive method for isoniazid determination in serum

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Abstract

An original simple, specific and rapid high-performance liquid chromatographic assay for the determination of isoniazid (INH) in human serum is presented. The drug was extracted from the serum by protein precipitation with 30% (w/v) trichloroacetic acid, then the drug was reacted with the coupling reagent, *trans*-cinnamaldehyde, to form a derivative absorbing at 340 nm. A 20-μl aliquot was injected into the chromatograph after neutralization with 1 M KOH solution. A liquid chromatograph equipped with a reversed-phase 30-μm C_{18} precolumn linked to a 4-μm C_{18} analytical column was used. The drug was eluted with a mixture of acetonitrile–water–triethylamine–acetic acid (400:600:2:1, v/v), pH value was 5 ± 1. Flow-rate and wavelength were set at 1 ml/min and 340 nm, respectively. The extraction recoveries from human serum averaged 100% for INH at concentrations of 1, 2 and 4 mg/l. The coefficients of variation for three different concentrations of INH in serum in the within-day study varied between 1.2 and 3.5%, whereas those in the day-to-day study varied between 2.8 and 4.3%.

Keywords: Isoniazid

1. Introduction

The acquired immunodeficiency syndrome (AIDS) increases the frequency of pulmonary tuberculosis [1]. Presently, tuberculosis is the cause of death of about 1000 people in France and 3 000 000 in the world per year [1]. Isoniazid (INH) is an antitubercular drug which has been classified as a first-line drug in spite of its toxicity. The importance of this drug is due to its wide-spread use and its efficiency in the treatment of pulmonary tuberculosis.

Both toxic effects or lack of therapeutic effect of INH involve the need for quantification of the drug by a suitable and rapid technique. Non-chromatographic assays developed for INH determination in serum and plasma included spectrophotometric [2] and spectrofluorometric [3] procedures. These procedures are often time-consuming and tedious. HPLC methods for the estimation of INH involving liquid–liquid extraction [4–7] or solid-phase extraction [8] are laborious and poorly sensitive.

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This paper describes an original method for the INH assay in human serum using a simple protein precipitation with 30% trichloroacetic acid and coupling with *trans*-cinnamaldehyde. The determination of INH concentration by HPLC uses the external standard procedure.

2. Experimental

2.1. Reagents

Analytical-grade triethylamine, trichloroacetic acid and HPLC-grade acetonitrile were purchased from Merck (Darmstadt, Germany). Isoniazid and acetylisoniazid were a generous gift from Hoffmann-La Roche Lab. (Basel, Switzerland). *Trans*-cinnamaldehyde was purchased from Fluka (Buchs, Switzerland). A stock solution of 0.1% *trans*-cinnamaldehyde was prepared by diluting 1000-fold the *trans*-cinnamaldehyde pure solution with methanol to give a final concentration of 0.1 ml per 100 ml.

2.2. Sample preparation

Three external standards containing 1, 2 and 4 mg/l of INH were prepared from a 10 mg/l INH solution (50, 100 and 200 μl of this solution filled to 500 μl with INH-free serum). Samples of serum (0.5 ml) were precipitated with 100 μl of 30% (w/v) trichloroacetic acid. They were mixed and then centrifuged for 5 min at 2000 g. An aliquot of 20 μl of 0.1% of the coupling reagent *trans*-cinnamaldehyde was added to 100 μl of the supernatant. After 10 min, 20 μl of 1 M KOH solution were added in order to obtain a pH range between 4 and 6.

2.3. Chromatography conditions

The HPLC system consisted of a Chromatem 380 high-pressure solvent delivery system (Touzart & Matignon, Vitry-sur-Seine, France) equipped with a Model 7125 Rheodyne valve and fitted with a Nova-pak C_{18} column (125 × 3.9 mm I.D., 4 μm particle size) from Waters (Milford, MA, USA). Absorbance of the derivative

resulting from the coupling of INH with *trans*-cinnamaldehyde was measured at 340 nm with 0.16 AUFS using a Spectroflow 757 (Kratos, Ramsey, NJ, USA) UV detector. The mobile phase was a mixture of water–acetonitrile–triethylamine–acetic acid (600:400:2:1), and pH was 5 ± 1. The UV response is constant within this pH range as for the retention times of INH and blank peaks. Flow-rate was set at 1.3 ml/min. Chromatograms were recorded on a BD41 recorder (Kipp & Zonen, Delft, Netherlands) and the peak heights were measured.

2.4. Calibration and accuracy

Calibration curve was constructed by adding known quantities of INH to serum and plotting INH peak heights against the amount of INH added. The standard deviation of the normalized peak heights was used to determine the accuracy of the method over the range of INH standards used.

The precision of the method was also studied by submitting 10 replicate serum samples containing 1, 2 and 4 mg/l INH to the entire procedure. To estimate the recoveries with this method, the peak heights of 0.5-ml samples containing known amounts of INH were compared to the respective peak heights obtained by injecting equal concentrations directly into the chromatograph.

3. Results

Fig. 1 shows the chromatograms obtained from the serum extract of non-treated recipients and treated recipients, respectively. The retention times of coupled INH and *trans*-cinnamaldehyde reagent in excess were 1.95 min and 3.50 min, respectively.

3.1. Linearity

Sample concentrations were calculated by measuring the peak heights with reference to the peak heights of increasing amounts of INH from the standard solution. There is a linear relation-

(A)

Isoniazid

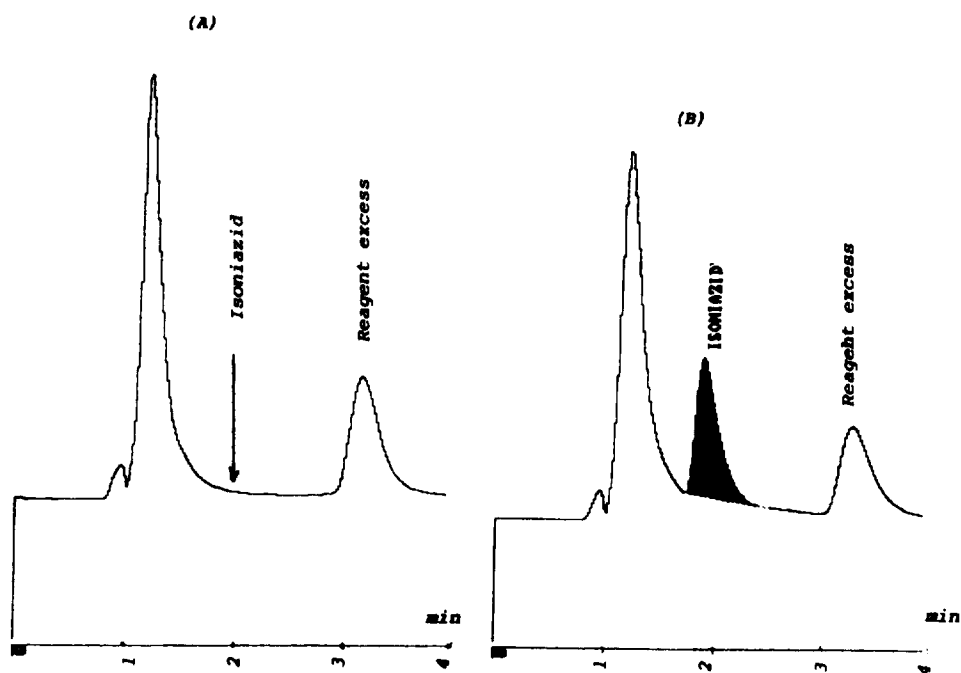Reagent excess

(B)

ISONIAZID

Reageht excess

min

min

Fig. 1. Chromatograms obtained after analysis of human serum by the recommended procedure. (A) Human free-isoniazid serum. (B) Patient sample treated with INH (final concentration: INH = 1.72 mg/l).

ship between peak heights and concentrations: $r = 0.999$ for 0.2 to 10 mg/l INH range.

3.2. Recovery

Recovery was studied by adding known amounts of INH to the serum of an untreated person. After extraction of the drug, concen-

trations and recoveries were calculated (Table 1).

3.3. Lower detection limit

Under the operating conditions described above and for 0.5 ml of serum sample, the detection limit was 0.02 mg/l. The limit of

Table 1
Recovery of isoniazid from human after protein precipitation

Concentration added (mg/l)	Concentration found (mean ± S.D., $n = 10$) (mg/l)	Coefficient of variation (%)	Recovery (%)
Within-day variation			
1	1.01 ± 0.03	3.5	101.1
2	2.09 ± 0.03	3.6	104.5
4	4.02 ± 0.01	1.2	100.5
Day-to-day variation			
1	0.99 ± 0.04	4.3	99.1
2	2.04 ± 0.03	3.2	102.2
4	3.99 ± 0.22	2.8	99.7

detection usually reported for LC methods is 0.1 mg/l. Therapeutic concentrations of INH range between 1 and 2 mg/l.

3.4. Specificity

The main metabolite of INH, acetylisoniazid, did not react with *trans*-cinnamaldehyde and did not absorb at 340 nm.

None of the following drugs often given to patients receiving INH therapy interfered with the estimation of INH: streptomycin, rifampicin, pyrazinamide and pyridoxine. The following drugs (concentration tested for each: 5 mg/l) which represent different therapeutic classes, could be administered in association with INH: aceprometazine, adrafinil, alimemazine, allopurinol, alprostadil, altretamine, baclofen, bendroflumethiazide, benserazide, betamethasone, bisoprolol, bromocriptine, caffeine, captopril, chlorpromazine, clomipramine, clonazepam, cortisone, cyamemazine, difebarbamate, dosulepin, ethosuximide, fenspiride, flumazenil, fluoxetin, fluvoxamine, halofantrin, hydrochlorothiazide, hydroxyzine, ibuprofen, imipramine, levamisol, levodopa, maprotilin, medifoxamine, metopimazine, midazolam, naftazone, naftidrofuryl, naproxen, nicergolin, nitrazepam, nordazepam, nortriptylin, penfluridol, phenobarbital, pimozide, pipamperone, pipotizine, primidone, quinine, selegilin, tenormine, tetrazepam, theophylline, thioproperazide, tiaprid, triazolam, trihexyphenidyl, trimipramine, tropatepine, vigabatrin and zopiclone. No analytical interference was observed.

4. Discussion

Initially, our aim was to assay INH in human serum using an HPLC method described elsewhere. However, we have met difficulties, particularly in the extraction phase: all extraction procedures reported were tedious and/or time-consuming. Extraction procedures most often used are liquid–liquid extractions and generally consist of two steps or more. In the first step, the drug is extracted from biological fluids by organic solvents (butanol, dichloromethane, diethyl ether, acetone or other) with a suitable pH value, generally basic. The second step consists in the reextraction of INH from the organic phase by an aqueous acidified solution. A solid-phase extraction clean-up was reported by Gartonde and Pathak [8], but we have not been successful in obtaining their results.

The low sensitivity of INH, the difficulty to easily separate INH from its main metabolite acetylisoniazid and the duration of the different extraction procedures published up to now, encouraged us to develop a new method for INH determination.

All extraction procedures for INH reported until now need specimen "cleanup" (e.g. back-extraction). In our extraction procedure, there was no such "cleanup" because very few drugs and endogenous substances absorb at 340 nm; therefore our extraction procedure came down to a simple protein precipitation. Until now, we did not find an internal standard which can react with *trans*-cinnamaldehyde and possessing the same chemical properties as INH. The reproducibility and recovery results obtained with an external standard were very satisfactory (see Table 1). After precipitation, the samples can be processed or stored at 4°C in order to avoid evaporation. Recoveries were greater than 95% at concentrations of 0.1 mg/l to 10 mg/l.

The condensation of INH and *trans*-cinnamaldehyde by their hydrazide group and aldehyde group, respectively, should result in a hydrazone with the formula outlined in Fig. 2. Eidus and Harnanansingh were the first who reported the use of *trans*-cinnamaldehyde as coupling reagent to estimate INH in serum or plasma. Their spectrophotometric method is sensitive but tedious [9]. There is no reaction with acetylisoniazid, the main metabolite of INH, because of its acetyl
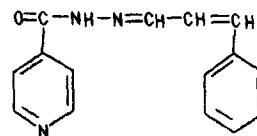

Fig. 2. Possible formula of the derivative resulting from the coupling of *trans*-cinnamaldehyde with isoniazid.

group which is linked to INH-hydrazide group. However, acetylisoniazid has no toxic or therapeutic effects. Its determination is not necessary except in phenotype determination cases or in pharmacokinetic studies. The determination of INH alone is sufficient to adapt the dosage. To calculate the dosage, we used, with some minor modifications, the equation of Vivien et al. [10]:

$$Dosage\ advised = \frac{2.1\ a}{0.6 + b}\ \text{mg kg}^{-1}\ \text{day}^{-1}$$

a = the dosage of INH in mg kg^{-1} day^{-1}, and b = the INH concentration in mg/l 3 h after oral administration. The calculation is based on the fact that the therapeutic concentration of INH 3 h after oral administration is 1.5 mg/l. Of course, to estimate the dosage exactly, we must include in the equation the apparent volume of distribution of INH [11].

4. Conclusions

With the method described herein, 80 samples can be processed in a working day. The HPLC analysis is sensitive and selective and does not involve complex combinations of organic solvents in the mobile phase. The sample volume required is only 0.5 ml compared to 1 or 2 ml for other methods. This method for the estimation of INH in serum is considerably more sensitive than all previous chromatographic and spectrophotometric methods. We have performed more than 1000 determinations with this method.

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