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DETERMINATION OF ISONIAZID, ACETYLISONIAZID, ACETYLHYDRAZINE AND DIACETYLHYDRAZINE IN BIOLOGICAL FLUIDS BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A high-performance liquid chromatographic assay for the determination of isoniazid, acetylisoniazid, acetylhydrazine and diacetylhydrazine (plasma and urine) was developed. The *m*-chlorobenzoyl derivatives of isoniazid, acetylhydrazine and the internal standard propionylhydrazine were prepared, separated on a RP-18 column and detected at 220 nm. Acetylisoniazid, diacetylhydrazine and the internal standard dipropionylhydrazine were converted to isoniazid, acetylhydrazine, and propionylhydrazine by acidic hydrolysis and subsequently derivatized with *m*-fluorobenzoyl chloride, separated on a RP-18 column and detected at 220 nm. The lower limits of detection in plasma are acetylhydrazine 0.5 nmol/ml, isoniazid 1.0 nmol/ml, diacetylhydrazine 1.0 nmol/ml and acetylisoniazid 2.0 nmol/ml, and in urine, acetylhydrazine 10 nmol/ml, isoniazid 15 nmol/ml, diacetylhydrazine 20 nmol/ml and acetylisoniazid 40 nmol/ml. This method is sensitive, reproducible, accurate and precise; therefore, it is well suited for detailed pharmacokinetic studies.

INTRODUCTION

Isoniazid (INH, isonicotinic acid hydrazide) is a major drug used in the treatment of tuberculosis. A major drawback to its use is the frequent occurrence of hepatotoxicity [1, 2]. Animal studies have demonstrated that the hepatotoxicity of isoniazid is caused by acetylhydrazine which is formed by hydrolysis of the major INH metabolite acetylisoniazid. Apart from the N-acetylation of acetylhydrazine to diacetylhydrazine, it is further metabolized by a cytochrome P-450 dependent reaction. In this reaction a reactive acyl radical or acyl cation is formed which binds covalently to liver macromolecules, an event that may cause liver cell necrosis [2—4]. The higher incidence of INH-related hepatotoxicity in rapid acetylators has been attributed to their higher

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rate of formation of acetylhydrazine from acetylisoniazid [1, 2]. Recent studies have definitely identified acetylhydrazine as a metabolite of INH in man [5]. However, no detailed studies on the pharmacokinetics of INH and its hydrazine metabolites have been performed in patients to determine if formation and metabolism of acetylhydrazine might be correlated with isoniazid hepatotoxicity in man. Such studies are difficult to perform because methods with high sensitivity and specificity were not available for quantification of INH and its hydrazine metabolites in plasma and urine. Older methods employed the formation of the corresponding Schiff base and required the separate derivatization and determination of each compound [6]. As stated by the authors, this method was only applicable for the measurement of INH and hydrazine metabolites after single-dose administration. A specific gas—liquid chromatographic method for the determination of INH and hydrazine metabolites in urine has been reported [7]. This method, however, requires large volumes of organic solvents (600 ml), is time-consuming and the internal standard (the *p*-fluorobenzaldehyde derivative) is added after derivatization. Recently, a specific and sensitive gas chromatographic—mass spectrometric (GC—MS) method using single-ion monitoring (SIM) has been described [8]. Although SIM is, at present, the most specific method available, it requires access to a GC—MS system, which is not always readily available.

We describe a high-performance liquid chromatographic (HPLC) method which allows for the simultaneous determination of INH and its hydrazine metabolites in plasma and urine after formation of the corresponding hydrazides with halogen-substituted benzoyl chlorides, followed by separation on a reversed-phase column and UV detection. This method has been satisfactorily used by us during the last two years to measure INH and its hydrazine metabolites with good specificity, sensitivity, accuracy and precision.

MATERIALS AND METHODS

Synthesis of reference compounds and internal standards

INH was obtained from Bayer (Leverkusen, F.R.G.) and was recrystallized in methanol—diethyl ether (1:4). The melting point was 171—173°C [9].

Acetylhydrazine (AH) was synthesized by reacting a four-fold excess of hydrazine hydrate with the methyl ester of acetic acid. The reaction was allowed to proceed for 12 h at room temperature with continuous stirring. The excess hydrazine was removed by vacuum distillation. Acetylhydrazine was recrystallized in dichloromethane—*n*-hexane (2:1). The melting point of the synthesized product was 68—70°C (lit. [10]: 67°C).

Diacetylhydrazine (DAH) was synthesized by reacting hydrazine hydrate with a four-fold excess of the methyl ester of acetic acid at room temperature with continuous stirring for 12 h. Diacetylhydrazine was recrystallized in dichloromethane—*n*-hexane (1:4). The melting point of the synthesized product was 133—136°C (lit. [11]: 138—143°C).

Acetylisoniazid (AINH) was synthesized by reacting isoniazid with a four-fold excess of acetic acid anhydride at room temperature for 1.5 h with continuous stirring. The synthesized product was recrystallized in methanol—diethyl ether (1:4). The melting point of the final product was 157—160°C (lit. [12]: 162—163°C).

Propionylhydrazine (PH) was synthesized by reacting a four-fold excess of hydrazine hydrate with the methyl ester of propionic acid. The reaction conditions were the same as described for the synthesis of acetylhydrazine. The final product was distilled under vacuum. The melting point was 37°C (lit. [13]: 40°C).

Dipropionylhydrazine (DPH) was synthesized in the same way as described for the synthesis of diacetylhydrazine using the methyl ester of propionic acid instead of the methyl ester of acetic acid. The melting point of the synthesized product was 137—139°C (lit. [14]: 136°C).

3-Chlorobenzoyl chloride and 3-fluorobenzoyl chloride were purchased from EGA Chemie and purified by distillation under vacuum.

Apparatus

The liquid chromatographic system consisted of a Spectra-Physics 3500 B liquid chromatograph coupled to a Spectra-Physics Model 770 spectrophotometer. Detection was at 220 nm. A 300 mm (6 mm O.D. and 4.5 mm I.D.) stainless-steel column packed with 10-μm Nucleosil RP-18 (Macherey, Nagel & Co., Düren, F.R.G.) was used.

HPLC system A. The 3-chlorobenzoyl derivatives were separated with the following mobile phase: 10 m*M* phosphate buffer pH 5.5—methanol—acetonitrile—dichloromethane (73:17:9:1) at a flow-rate of 2 ml/min at 50°C.

HPLC system B. For the separation of 3-fluorobenzoyl derivatives (after hydrolysis) the mobile phase consisted of 10 m*M* phosphate buffer pH 5.1—acetonitrile—dichloromethane (84.2:15:0.8). The flow-rate was 2 ml/min at 50°C.

A LKB 2091 mass spectrometer (direct inlet system) was used to record the mass spectra.

The melting points were measured with a heating microscope (Zeiss/Reichert).

Analysis of INH and AH in plasma

The internal standards PH and DPH (20 nmol and 50 nmol, respectively, in 10 μl of acetonitrile) were added to 2 ml of plasma. Trichloroacetic acid (1 ml 20%) was added to precipitate plasma proteins. The sample was then centrifuged at 3000 *g* for 10 min. The supernatant was transferred to a test tube to which 20 μl of 0.1 *M* dipotassium hydrogen phosphate and 50 μl of 11 *M* sodium hydroxide were added to adjust the pH to 5—6. After 5 min of standing, interfering substances were extracted into 4 ml of dichloromethane—acetonitrile (9:1). Following centrifugation the aqueous phase (2 ml) was transferred to another test tube. Phosphate buffer (200 μl, 0.5 *M*, pH 4) was added and the pH adjusted to 2 with 11 *M* hydrochloric acid. Next, 3-chlorobenzoyl chloride (40 μmol; 200 μl of 0.2 *M* solution in acetonitrile) was added and the sample was allowed to react for 30 min at room temperature. The sample was then saturated with 1.4 g of sodium chloride and mixed with 4 ml of *n*-hexane for 30 sec on a vortex mixer. Following centrifugation at 3000 *g*, the organic phase was discarded and the sample was adjusted to pH 6—6.5 by addition of 0.45 ml of 1 *M* sodium bicarbonate and extracted into 4 ml of dichloromethane—acetonitrile (9:1) by mixing the sample for 30 sec on a vortex mixer.

After centrifugation, 3 ml of the organic phase were transferred to a tapered test tube and evaporated under a nitrogen stream. The remaining aqueous phase containing AINH, DAH and DPH was transferred to another test tube and used for the determination of AINH and DAH. The residue of the organic phase was dissolved in the mobile phase (0.2 ml; system A) and a 0.1-ml aliquot was injected onto the HPLC column. Mobile phase system A was used for the determination of INH and AH.

Analysis of AINH and DAH in plasma

The aqueous phase that had been used for the determination of INH and AH was extracted with 4 ml of dichloromethane—acetonitrile (9:1) to remove any remaining 3-chlorobenzoyl derivatives of isoniazid and acetylhydrazine. After centrifugation the organic phase was aspirated. To 1.5 ml of the aqueous phase 0.5 ml of 5 *M* hydrochloric acid was added and the sample was hydrolysed for 90 min at 50°C to transform AINH, DAH and DPH into INH, AH and PH. After hydrolysis phosphate buffer (0.2 ml; 0.5 *M*; pH 4) was added and the sample was adjusted to pH 2 with sodium hydroxide (450 µl; 2 *M*). 3-Fluorobenzoyl chloride (30 µmol; 0.10 ml of a 0.30 *M* solution in acetonitrile) was added and the sample was allowed to react for 30 min at room temperature. The sample was then saturated with sodium chloride (1.4 g), extracted with *n*-hexane (4 ml) for 30 sec on a vortex mixer, and centrifuged for 5 min at 3000 *g*. The aqueous phase containing the fluorobenzoyl derivatives formed from AINH, DAH and DPH was adjusted to pH 6—6.5 with 0.45 ml of 1 *M* sodium bicarbonate. After 5 min the derivatives were extracted into 4 ml of dichloromethane—acetonitrile (9:1). Following centrifugation the organic phase (3 ml) was transferred to a tapered test tube and evaporated to dryness under nitrogen. The residue was dissolved in mobile phase (0.2 ml; system B) and an aliquot (0.1 ml) was injected onto the column for determination of AINH and DAH.

Urine samples

For the determination of INH and the hydrazino metabolites in urine, the following procedure was used: internal standards PH and DPH (3 µmol; dissolved in 10 µl of acetonitrile) were added to urine (3 ml), then hydrochloric acid (30 µl; 11 *M*) was added. After 5 min the sample was extracted with dichloromethane—acetonitrile (9:1; 4 ml), centrifuged, the organic phase aspirated and the aqueous phase (2 ml) was transferred to another test tube. Subsequently the procedure was used as described for the plasma samples except for the following: (1) To each sample 60 µmol of 3-chlorobenzoyl chloride were added instead of 40 µmol. (2) The residue was dissolved in 2 ml of mobile phase (system A) and a 0.1-ml aliquot was injected onto the column. (3) For the determination of AINH and DAH 1 ml of the aqueous phase was hydrolysed with 1 ml of hydrochloric acid (2.5 *M*) under conditions described for plasma. (4) The residue was dissolved in 1 ml of mobile phase (system B) and a 0.1-ml aliquot was injected onto the column.

Standard curves

Quantification was done by use of the peak height ratio of 3-chlorobenzoyl-

isoniazid or 3-chlorobenzoyl-acetylhydrazine and 3-fluorobenzoyl-isoniazid or 3-fluorobenzoyl-acetylhydrazine to the internal standards 3-chlorobenzoyl-propionylhydrazine and 3-fluorobenzoyl-propionylhydrazine. A linear relation was obtained for the range tested. (Plasma: from the lower limit of detection (see Table I) to 50, 500, 100, and 500 nmol/ml for AH, INH, DAH, and AINH. Urine: from the lower limit of detection (see Table I) to 0.5, 5.0, 1.0, and 5.0 μmol/ml for AH, INH, DAH, and AINH.)

RESULTS

Figs. 1 and 2 show representative chromatograms of plasma and urine samples obtained from patients after the administration of INH. The compounds are well separated and no interfering peaks could be observed in blank plasma and urine samples. Retention times were 5.4, 6.9 and 9.1 min for AH, PH and INH, and 4.7, 6.2 and 9.2 min for DAH, DPH and AINH. Figs. 3 and 4 show the concentration—time curves of INH and its metabolites in plasma and urine.

Hydrolysis

Since the kinetics of acidic hydrolysis of AINH and DAH are different (Fig. 5), it was necessary to choose conditions such as temperature and time of the hydrolysis so that DPH could be used as a standard for both metabolites. Under the aforementioned conditions 60% AH and 40% INH were hydrolysed from DAH and AINH, respectively.

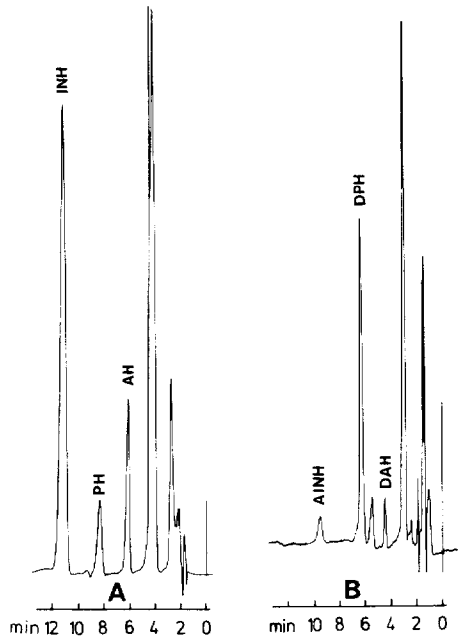

Fig. 1. Chromatograms of a plasma sample before (A) and after (B) acidic hydrolysis from a patient 3 h after intravenous administration of 10 mg/kg isoniazid. AINH = acetylisoniazid; AH = acetylhydrazine; DAH = diacetylhydrazine; DPH = dipropionylhydrazine; INH = isoniazid; PH = propionylhydrazine.

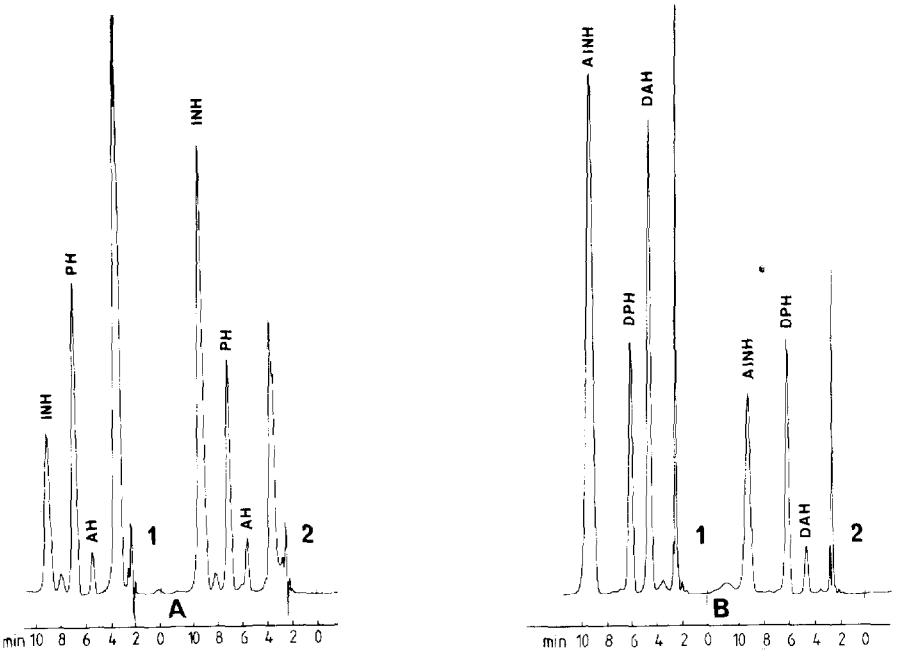

Fig. 2. Chromatograms of urine samples before (A) and after (B) acidic hydrolysis from a rapid (1) and a slow (2) acetylator after intravenous administration of 10 mg/kg isoniazid. Abbreviations as in Fig. 1.

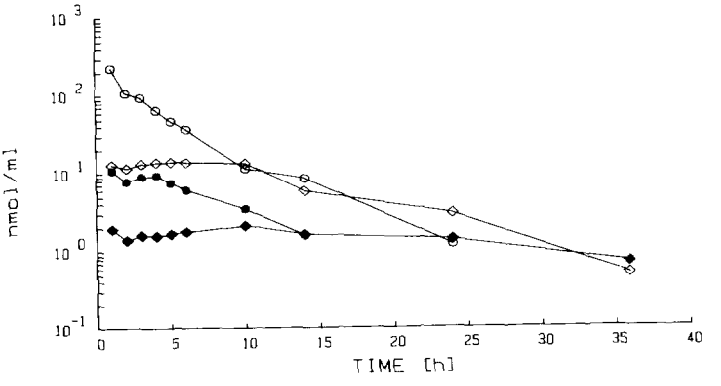

Fig. 3. Plasma level—time course of isoniazid (○), acetylhydrazine (◇), acetylisoniazid (●) and diacetylhydrazine (◆) following intravenous administration of 10 mg/kg isoniazid to a slow acetylator patient.

Sensitivity

The lower limits of detection in plasma and urine at a detector attenuation of 0.04 a.u.f.s. (signal-to-noise ratio of 5:1) are listed in Table I.

Precision

The precision for the determination of INH and its hydrazine metabolites was obtained by analysing pooled plasma and urine samples. The coefficients of variation (ten samples of each analysed) are listed in Table II.

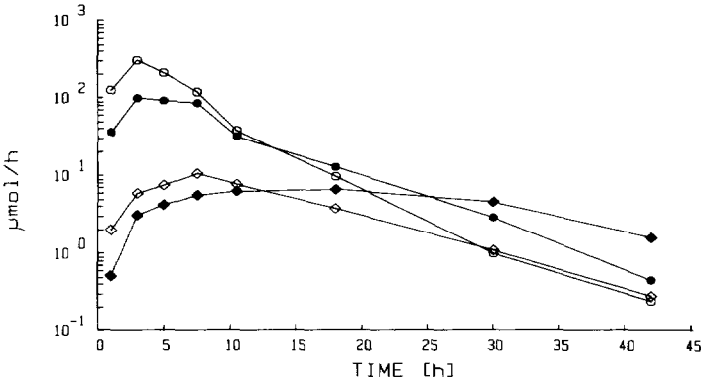

Fig. 4. Urinary excretion rates of isoniazid (○), acetylhydrazine (◇), acetylisoniazid (●) and diacetylhydrazine (◆) following intravenous administration of 10 mg/kg isoniazid to a slow acetylator patient.

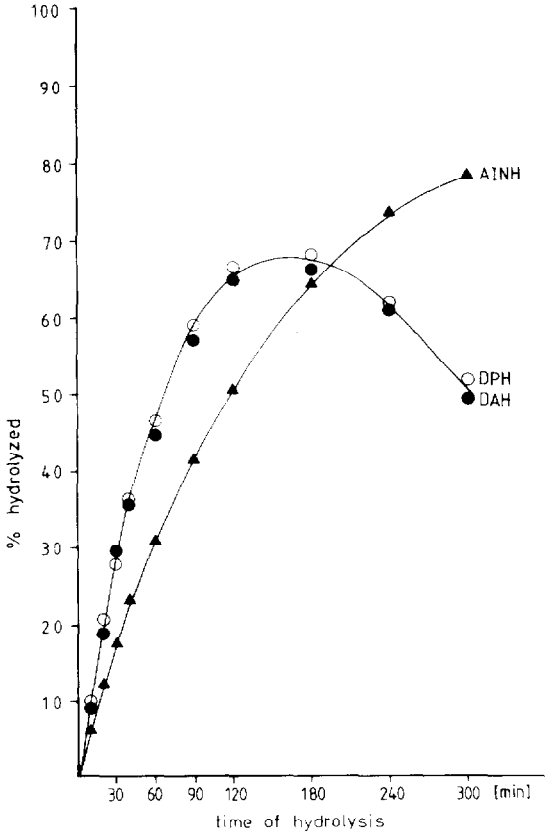

Fig. 5. Time course of the hydrolysis of diacetylhydrazine (●, DAH), dipropionylhydrazine (○, DPH) and acetylisoniazid (▲, AINH) to acetylhydrazine, propionylhydrazine and isoniazid.

TABLE I

LOWER LIMITS OF DETECTION FOR THE DETERMINATION OF ISONIAZID (INH), ACETYLHYDRAZINE (AH), ACETYLISONIAZID (AINH) AND DIACETYLHYDRAZINE (DAH) IN PLASMA AND URINE

Values are expressed in nmol/ml.

	INH	AH	AINH	DAH
Plasma	1.0	0.5	2.0	1.0
Urine	15.0	10.0	40.0	20.0

TABLE II

PRECISION OF THE DETERMINATION

Plasma and urine samples obtained from patients who were treated with isoniazid were pooled. Procedure 1: Ten samples of each pool were analysed on the same day. Procedure 2: Twelve samples of each pool were analysed over a period of six (plasma) or twelve (urine) months. Plasma concentrations (nmol/ml): INH, 22.0; AH, 10.1; AINH, 29.4; DAH, 4.8. Urine concentrations (nmol/ml): INH, 168.0; AH, 88.0; AINH, 1197.0; DAH, 147.0. The table shows the coefficients of variation (%).

	Procedure	INH	AH	AINH	DAH
Plasma	1	8.13	5.53	6.98	6.36
	2	8.59	6.39	8.79	8.51
Urine	1	1.61	1.36	4.02	4.05
	2	6.86	4.31	11.64	6.02

Day-to-day precision was obtained by analysing pooled urine and plasma samples on different days. For the plasma samples it was done within a period of six months, for urine samples within twelve months. The coefficients of variation are listed in Table II. In plasma and urine samples which were stored at −20°C no decrease in INH and metabolite concentrations could be observed over a period of six and twelve months, respectively.

Accuracy

The accuracy of the method was determined by adding known amounts of INH and INH metabolites to drug-free plasma and urine and analysing the samples as described. Tables III and IV show the results of these experiments. The coefficient of correlation found for plasma and urine was 0.99 for each compound.

Selectivity

Selectivity of this method was assessed by collecting the peaks of the INH, AH, AINH and DAH derivatives eluted from the column from plasma and urine samples of patients treated with isoniazid. The mass spectra of those peaks were identical with those obtained for reference compounds (direct inlet system). No other compounds could be detected during the complete evaporation of the samples.

TABLE III

ACCURACY OF THE DETERMINATION OF ACETYLHYDRAZINE (AH), ISONIAZID (INH), DIACETYLHYDRAZINE (DAH) AND ACETYLISONIAZID (AINH) IN PLASMA

Values are expressed in nmol/ml.

Sample	AH		INH		DAH		AINH	
	Added	Found	Added	Found	Added	Found	Added	Found
1	20	19.8	50	46.7	20	19.4	50	51.0
2	40	39.3	100	104.9	40	39.7	100	95.9
3	4	4.4	10	10.3	4	4.3	10	9.7
4	6	6.0	15	14.7	6	6.3	15	16.0
5	10	9.8	25	24.4	10	9.4	25	22.5
6	20	18.6	50	50.7	20	18.1	50	44.4
7	40	38.0	100	100.8	40	36.7	100	92.4
8	4	4.2	10	9.5	4	4.9	10	11.0
9	6	5.8	15	14.4	6	6.2	15	15.8
10	10	9.3	25	24.4	10	10.5	25	26.2

TABLE IV

ACCURACY OF THE DETERMINATION OF ACETYLHYDRAZINE (AH), ISONIAZID (INH), DIACETYLHYDRAZINE (DAH) AND ACETYLISONIAZID (AINH) IN URINE

Results are expressed as nmol/ml.

Sample	AH		INH		DAH		AINH	
	Added	Found	Added	Found	Added	Found	Added	Found
1	20	20.3	100	103.6	100	96.6	250	255.8
2	400	382.8	500	498.5	50	50.3	500	466.2
3	50	51.9	1000	985.5	100	94.8	150	141.5
4	200	183.9	500	500.0	100	110.9	1000	972.3
5	20	20.9	125	124.8	500	471.5	100	105.7
6	50	50.0	50	47.3	150	140.0	100	90.1
7	30	28.2	250	233.6	50	53.5	500	472.0
8	10	10.3	100	110.1	50	55.4	1000	962.7
9	200	182.9	50	48.4	100	100.4	1000	1083.1
10	100	97.3	500	494.0	200	187.8	500	546.0

DISCUSSION

The results indicate that the method described is sensitive, selective, precise and accurate. With the exception of the recently described method of Lauterburg et al. [8] which requires GC—MS, this is the only method which uses internal standards. The use of an internal standard is especially critical for the analysis of AINH and DAH. The measurement of these metabolites requires acidic hydrolysis to generate INH and AH which are then derivatized. In order to correct for differences in the kinetics of this hydrolysis reaction the use of an internal standard that is also hydrolysed is of the utmost importance.

In comparison to the use of aldehydes to form the corresponding imines — where only 90% is derivatized — derivatization with benzoyl chlorides yields a

nearly complete reaction (98%). A complete derivatization of INH and AH should be achieved, since non-derivatized INH and AH will interfere with the analysis of AINH and DAH. In adition, the benzoyl derivatives formed are stable in an aqueous solution at acidic pH in contrast to Schiff bases, which is of importance during the hydrolysis step since extraction of the derivatives is not complete. The use of two derivatizing reagents yields derivatives of different chromatographic behaviour and thus avoids interference from non-extracted derivatives formed before hydrolysis. Our initial attempt to separate and quantitate the chloro- and fluorobenzoyl derivatives in single-step chromatography was not possible, since one endogenous compound present in plasma and urine interfered with the determination of AINH.

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