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High-performance liquid chromatographic determination of isoniazid, acetylisoniazid and hydrazine in biological fluids

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Abstract

The basic principle of derivatization of a hydrazide moiety with an aldehyde as applied in the method developed by Lacroix et al. [J. Chromatogr., 307 (1984) 137–144] for the quantitation of isoniazid and acetylisoniazid was improved by modification, standardization and extension to allow quantitation of hydrazine in patient samples. It could be shown that 40 μl of 1% methanolic cinnamaldehyde per 200 μl of deproteinized analysate gave maximal chromophoric isoniazid–cinnamaldehyde conjugate, read at 340 nm. The hydrolytic loss of isoniazid, crucial to the quantitation of acetylisoniazid, could be compensated for by introduction of an appropriate set of calibration curves. Although the method described here allows quantitation of monoacetylhydrazine and diacetylhydrazine, in addition to hydrazine, in mono-spiked samples, the method cannot be used for the quantitation of the acetylated metabolites of hydrazine in patient samples because of a lack of specificity. Linear calibration curves in the range 1–25 μg/ml for isoniazid and acetylisoniazid, 10–400 ng/ml for hydrazine and 50–1000 ng/ml for monoacetylhydrazine and diacetylhydrazine, could be constructed; analyte recoveries approaching 100% could be achieved in all instances.

Keywords: Isoniazid; Acetylisoniazid; Hydrazine

1. Introduction

An HPLC method was sought with which to quantitate a wide range of concentrations of INH and AcINH accurately in small samples of biological fluids. Attempts to quantitate these polar compounds directly in unchanged form were unsatisfactory either because of unfavourable elution characteristics or because of error inducing extraction procedures or because of low analyte recovery rates [1–3]. Consequently, the method of Lacroix et al. [4], in which the hydrazide moiety of INH is derivatized with cinnamaldehyde to impart favourable elution and detection characteristics was evaluated. Additional potential advantages of the method in-

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clude: lack of error-inducing extraction procedures and high analyte recovery rates; high specificity for INH crucial to the quantitation of both INH and AcINH.

The full potential of the basic method developed by Lacroix could be realized by modification in which particular attention was paid to the standardization of analytical procedures, such as the derivatization and hydrolytic steps, and by introduction of a set of calibration curves to correct for the hydrolytic loss of INH crucial to the quantitation of AcINH. The modified method described here has the added advantage of allowing quantitation of hydrazine.

An inherent disadvantage of the basic method is that INH and AcINH cannot be quantitated simultaneously since AcINH has first to be converted to INH by hydrolysis, the increment in the molar concentration of INH being directly proportional to the molar concentration of AcINH.

2. Experimental

2.1. Reagents

trans-Cinnamaldehyde was "Gold Line" grade from Aldrich (Milwaukee, WI, USA) and contained less than 1% of cinnamic acid as impurity which did not interfere at the INH detection wavelength of 340 nm. Isoniazid was standard pharmaceutical grade from Fluka Chemie AG (Buchs, Switzerland) which contained 0.14% of hydrazine as impurity. AcINH was prepared by a standard method [5] and was shown by mass spectroscopy and melting point determinations to be more than 99% pure. Hydrazine was obtained from E. Merck (Darmstadt, Germany). Monoacetylhydrazine (MAcHz) and diacetylhydrazine (DAcHz) were obtained from Aldrich. Other reagents were "pro analysi" grade from E. Merck.

2.2. Chromatography

A Hewlett Packard 1090 L high-performance liquid chromatograph equipped with a Whatman Partecil 5 C_8 250 mm column (I.D. = 4.6 mm), maintained at 50°C, was used at a flow-rate of 1 ml/min. The mobile phase was composed of a mixture of solvent A (50 m*M* KH_2PO_4) and solvent B (acetonitrile–isopropanol; 4:1, v/v). For the first minute of chromatography the mobile phase contained 40% of solvent B, which was then increased linearly to reach 70% after 10 min, at which it was maintained until completion of the run at 14.5 min. For re-equilibration of the column 4 min was allowed between consecutive runs. Peaks were recorded at 340 nm and were quantified with an HP 3392 reporting integrator.

2.3. Sample management and preparation for chromatography

Venous blood was collected into pre-cooled EDTA-coated tubes and was immediately centrifuged at 0°C; the samples were chilled on ice prior to further processing. Fig. 1 outlines the procedures followed in the preparation of samples. Where required, all acetylated compounds (AcINH, MAcHz and DAcHz) were hydrolytically deacetylated with 20 μl 6 *M* HCl per 200 μl of analysate maintained at 80°C for 1 h.

2.4. Concentrations and concentration ranges studied

For the evaluation of the effects of hydrolyzing conditions on the analytical yield of INH from

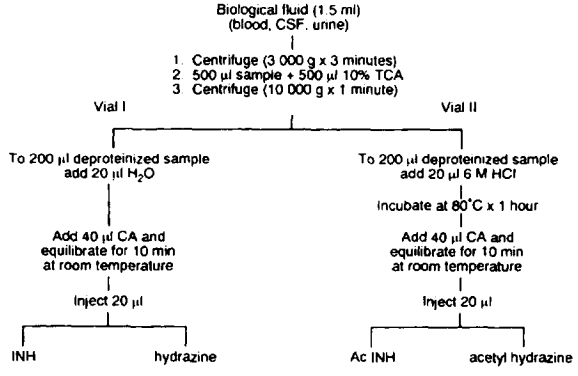

Fig. 1. Flow diagram of preparative procedures prior to HPLC analysis.

matrix INH and from AcINH, as per Fig. 4, plasma samples were spiked with 10 and 13.065 μg/ml of INH and AcINH, respectively, to ensure equimolarity since M_r AcINH:M_r INH = 1.3065.

For the construction of the calibration curves, shown in Figs. 5 and 6, drug-free plasma samples were spiked with INH and AcINH (range: 1–25 μg/ml), Hz (range: 10–400 ng/ml), MAcHz and DAcHz (range: 10–1000 ng/ml). Calibration curves were constructed from appropriately spaced concentration points covering the ranges indicated above: INH and AcINH-8 points; Hz-6 points; MAcHz-8 points and DAcHz-7 points. Each concentration point was taken as the mean of five repeat analyses and each calibration curve was constructed on five different occasions. In the construction of the calibration curves of INH and AcINH, equimolarity was ensured by using 1.3065 mass units of AcINH per mass unit of INH indicated in the abscissa of Fig. 5, where applicable. In the construction of the calibration curves of Hz, MAcHz and DAcHz, equimolarity was ensured by using 2.3118 mass units of MAcHz and 3.6236 mass units of DAcHz per mass unit of Hz indicated in the abscissa of Fig. 6, where applicable, since M_r MAcHz:M_r Hz = 2.3118 and M_r DAcHz:M_r Hz = 3.6236.

In order to assess analytical recovery rates in samples containing both INH and AcINH, plasma samples were spiked with varying relative concentrations of both compounds, as indicated in Table 2, to cover the concentration ranges usually occurring in patient samples.

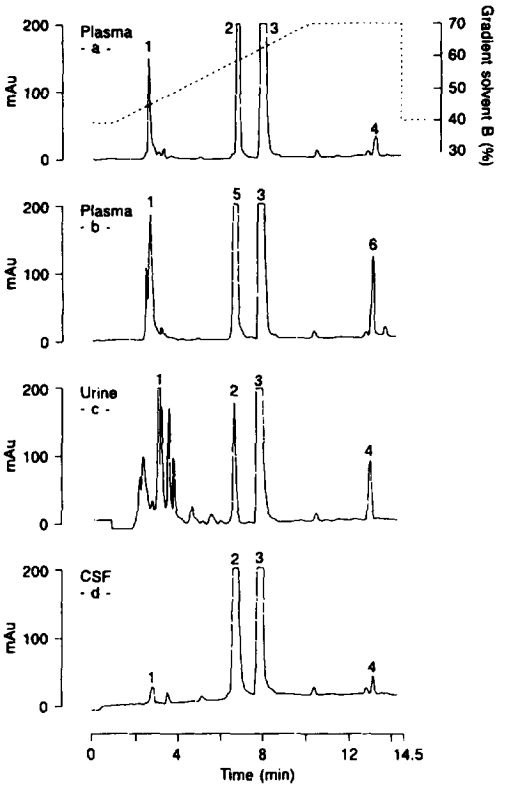

Fig. 2. HPLC chromatograms of derivatized INH and Hz in a plasma sample from a patient, before and after hydrolysis, are shown in traces marked a and b, respectively. The profiles of INH and Hz in urine and CSF are also shown, traces marked c and d, respectively. The gradient composition in terms of solvents A and B, over the time course of a run, is indicated by the dotted line. The traces show the peaks (numbered) generated by similarly numbered compounds: 1 = miscellaneous impurities; 2 = free INH; 3 = cinnamaldehyde (CA); 4 = free hydrazine (Hz); 5 = total INH post-hydrolysis; 6 = total hydrazine post-hydrolysis.

3. Results

Chromatograms of non-hydrolyzed and hydrolyzed plasma are shown in Fig. 2; trace a reflects the profile of a sample containing INH (7.16 μg/ml) and Hz (26.7 ng/ml) pre-hydrolysis and trace b reflects the profile of the same sample containing INH (15.31 μg/ml) and Hz (73.7 ng/ml) post-hydrolysis. Chromatograms of unhydrolyzed urine and CSF are also shown in Fig. 2; trace c reflects the profile of INH (2.47 μg/ml) and Hz (53.8 ng/ml) in urine and trace d reflects the profile of INH (11.71 μg/ml) and Hz (22.8 ng/ml) in CSF.

Fig. 3 shows that the yield of INH–CA complex reaches a maximum value after addition of 30 to 40 μl of a 1% methanolic solution of CA per 200 μl of supernatant or analysate in contrast to the 10 μl of solution per 200 μl of analysate used by Lacroix et al. [4]. On the basis of the foregoing, 40 μl of 1% cinnamaldehyde per 200 μl analysate was chosen as standard for all derivatization procedures in order to accommodate varying concentrations of INH and AcINH

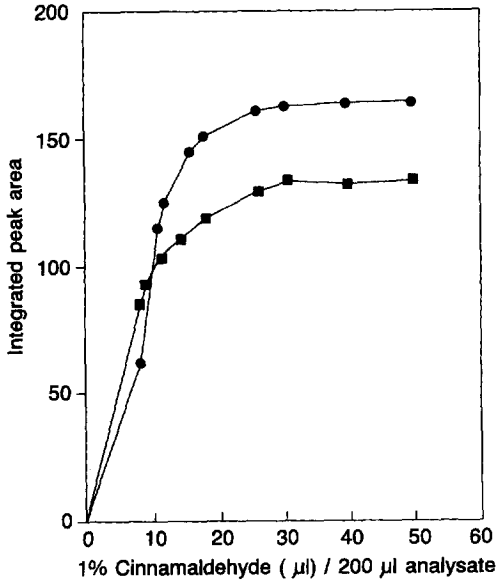

Fig. 3. Integrated peak area of 10 μg/ml of INH (●) and of INH hydrolytically liberated from 13.065 μg/ml of AcINH (■) expressed as a function of the volume of derivatizing solution (1% cinnamaldehyde in methanol) per 200 μl of analysate. Volume constancy was effected with methanol.

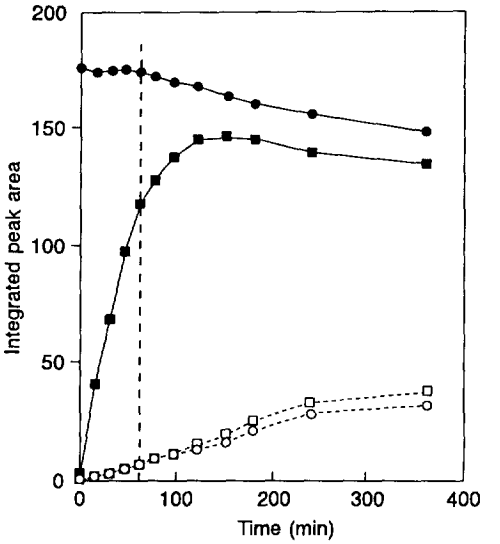

Fig. 4. Integrated peak area of free INH (10 μg/ml) subjected to hydrolyzing conditions (●) and INH liberated from equimolar AcINH (13.065 μg/ml) by ongoing hydrolysis (■), are shown as a function of time (ratio M_r AcINH:M_r INH = 1.3065). Hydrazine generation from hydrolyzed INH (○) and hydrolyzed AcINH (□), as a function of time, is also shown.

in patient samples. The cinnamaldehyde used in derivatization (12.2 mM CA) yielded approximately 800 integrator area units and was unaffected by addition of 20 μl of 6 M HCl prior to the hydrolytic step.

It is evident from Fig. 4 that equimolar concentrations of INH and AcINH—[INH] (10 μg/ml):[AcINH] (13.065 μg/ml)—subjected to hydrolyzing conditions for 1 h do not yield equal concentrations of INH (basis for chromophoric complex) making accommodation of the differences in an appropriate set of calibration curves a prerequisite to AcINH quantitation in patient samples containing both INH and AcINH. It is also evident that Hz is formed during the hydrolysis of both INH and AcINH.

All calibration plots shown in Figs. 5 and 6 indicate direct proportionality between the concentration of the analyte and absorbance. In no instance was the intra-sample CV of the concentration values >2% for INH or AcINH, >than 3.25% for Hz, >2.75% for MAcHz and >2.5% for DAcHz, and in no instance was the

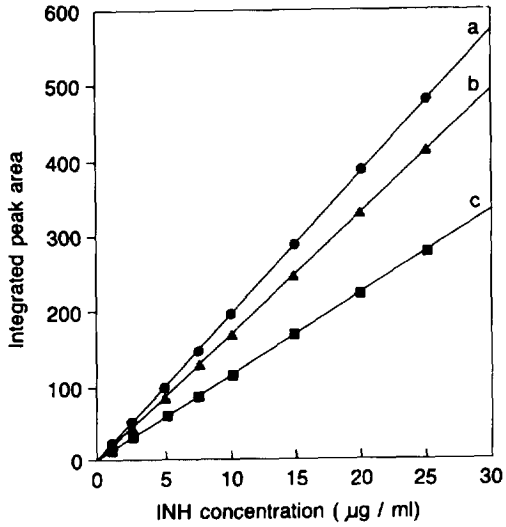

Fig. 5. Calibration curves are shown for free INH not subjected to hydrolyzing conditions (plot a, $r = 0.99979$), INH subjected to hydrolyzing conditions (plot b, $r = 0.99919$) and INH hydrolytically liberated from equimolar AcINH (plot c, $r = 0.99991$).

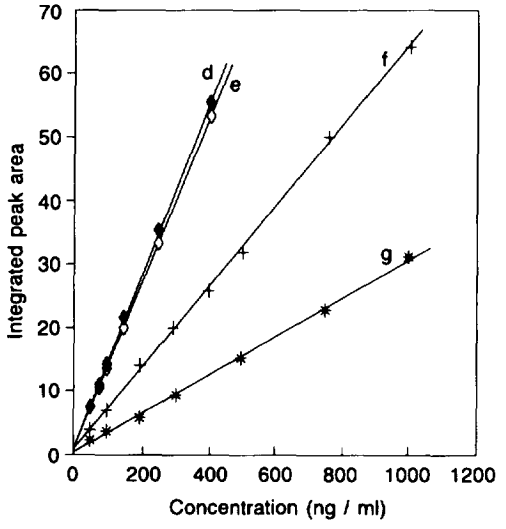

Fig. 6. Calibration curves are shown for hydrazine (plot d, $r = 0.99993$) not subjected to hydrolyzing conditions and hydrazine (plot e, $r = 0.99984$), MAcHz (plot f, $r = 0.99905$) and DAcHz (plot g, $r = 0.99894$), all subjected to hydrolyzing conditions.

inter-sample CV >2% for INH or AcINH, and >3.5% for Hz, >3.5% for MacHz and >3% for DAcHz. The standard curves in respect of INH, AcINH and Hz in CSF and urine were identical to those generated in plasma (data not shown). The detection limits were 0.5 μg/ml for INH and AcINH and 10 ng/ml for Hz, MAcHz and DAcHz, at a 95% confidence level.

It is evident from the slopes of the respective calibration plots, shown in Fig. 5, that the yield of INH, measured as INH–CA complex, from equimolar concentrations of INH and AcINH, was different for INH not subjected to hydrolyzing conditions (19.4959 ± 0.5070 absorption units (AU)/μg INH/ml plasma, $r = 0.99979$, 8 concentration points, $n = 5$ per data point), INH subjected to hydrolyzing conditions (16.4198 ± 0.4657 AU/μg INH/ml plasma, $r = 0.99919$, 8 concentration points, $n = 5$ per data point) and AcINH subjected to hydrolyzing conditions (11.3693 ± 0.3207 AU/μg INH/ml plasma, $r = 0.99991$, 8 concentration points, $n = 5$ per data point). Calibration data for INH and AcINH in urine and CSF were identical to those in respect of these substances in plasma (data not shown). Consequently, calibration curves generated in plasma could be used for the quantitation of INH and AcINH in all biological fluids—plasma, CSF and urine. As shown in Table 1 the recovery rates of INH and AcINH in both plasma and urine, based on a common set of calibration curves, approached 100%.

Data in respect of hydrazine in spiked plasma and urine samples are also shown in Table 1. The calibration curve for Hz in plasma over the concentration range of 10 to 400 ng/ml was linear ($r = 0.99993$, 6 concentration points, $n = 5$ per data point) and was not affected by hydrolyzing conditions—graphs marked d and e in Fig. 6. Calibration curves for MAcHz and DAcHz in plasma were also linear: MAcHz-$r = 0.99905$, 8 concentration points, $n = 5$ per data point; DAcHz-$r = 0.99894$, 7 concentration points, $n = 5$ per data point; graphs marked f and g in Fig. 6 refer. Calibration curves for Hz, MAcHz and DAcHz generated in urine and CSF were essentially the same as those generated in plasma—data for Hz in spiked plasma and urine samples are shown in Table 1.

From these results it is clear that free INH and Hz concentrations can be determined directly by dividing the absorbance units by the slope of the applicable calibration curve. The AcINH concentration can be calculated by using the slopes (denoted by S) of the calibration curves of INH-free ab initio, not subjected to hydrolyzing conditions, INH-free ab initio, subjected to hydrolyzing conditions, and liberated INH, ex AcINH, subjected to hydrolyzing conditions (Sa, Sb and Sc, from plots a, b and c in Fig. 5, respectively) and the corresponding situational absorbances (Aa, Ab and Ac) using the formula: $[\text{AcINH}] = [(\text{Ab} + \text{Ac}) - \text{Ab}^*]\ (1.3065/\text{Sc})$, in which $\text{Ab}^* = \text{Aa}\ (\text{Sb}/\text{Sa})$ and $1.3065 = M_r\ \text{AcINH}/M_r\ \text{INH}$. Ideally $\text{Ab} = \text{Ab}^*$.

The recovery rates of analyte from samples spiked with both INH and AcINH are shown in Table 2 and are satisfactory for INH for the entire concentration range which was studied. For reasons which become apparent on inspection of the formula for the quantitation of AcINH, the accuracy and the reproducibility of AcINH values will depend on the relative con-

Table 1
Analytical recovery values of INH, AcINH and Hz from spiked plasma and urine samples

[Analyte][a] (μg/ml)	[INH][b] (μg/ml)	Recovery (%)	[AcINH][b] (μg/ml)	Recovery (%)
Analyses on spiked plasma (n = 5 per concentration point)				
1.0	1.02 ± 0.017	102.10	1.07 ± 0.031	107.06
2.5	2.52 ± 0.034	100.84	2.60 ± 0.029	104.09
5.0	5.14 ± 0.051	102.82	5.19 ± 0.064	103.81
7.5	7.49 ± 0.079	99.97	7.58 ± 0.089	101.17
10.0	10.09 ± 0.088	100.98	9.87 ± 0.176	98.74
15.0	15.03 ± 0.131	100.17	14.97 ± 0.142	99.80
20.0	19.89 ± 0.156	99.46	20.20 ± 0.118	101.00
25.0	25.00 ± 0.165	100.01	24.92 ± 0.173	99.66
Analyses on spiked urine (n = 5 per concentration point)				
2.5	2.54 ± 0.022	101.76	2.46 ± 0.161	98.25
5.0	4.92 ± 0.118	98.30	4.91 ± 0.094	98.18
10.0	9.67 ± 0.202	96.74	9.92 ± 0.106	99.19
15.0	14.77 ± 0.103	98.47	14.68 ± 0.153	97.87
20.0	19.22 ± 0.379	96.17	19.06 ± 0.198	95.30
25.0	25.36 ± 0.318	101.44	24.34 ± 0.235	97.37
[Analyte][a] (ng/ml)	[Hz][b] (p) (ng/ml)	Recovery (%)	[Hz][b] (u) (ng/ml)	Recovery (%)
Analyses on spiked plasma (p) and urine (u) (n = 5 per concentration point)				
10.0	9.65 ± 0.02	96.56	10.51 ± 0.06	98.25
50.0	49.29 ± 0.42	98.30	49.18 ± 0.78	98.18
75.0	72.18 ± 1.32	96.74	73.01 ± 1.86	99.19
100.0	96.43 ± 1.64	98.47	94.78 ± 2.34	97.87
150.0	146.51 ± 2.52	96.17	145.63 ± 4.19	95.30
250.0	241.48 ± 7.54	96.59	246.63 ± 8.46	98.65
400.0	388.96 ± 9.08	97.24	392.74 ± 9.61	98.18

[a] and [b] indicate the magnitude of the concentration of the spiked analyte and the corresponding analytical value, respectively.

Table 2
Recovery values of analyte from spiked plasma samples with varying relative content of INH and AcINH

[Analyte][a] composition (μg/ml)		n	Analytical result (μg/ml)		Recovery (%)	
[INH]	[AcINH]		[INH][b]	[AcINH][b]	[INH][b]	[AcINH][b]
1.0	9.0	5	0.985 ± 0.018	9.047 ± 0.153	98.52	100.52
2.5	7.5	5	2.572 ± 0.008	7.793 ± 0.081	102.88	103.91
5.0	5.0	5	5.114 ± 0.020	4.954 ± 0.042	102.28	99.08
7.5	2.5	5	7.709 ± 0.078	2.377 ± 0.050	102.78	95.08
9.0	1.0	5	9.047 ± 0.015	0.825 ± 0.157	100.52	82.54

[a] and [b] indicate the magnitude of the concentration of the spiked analyte and the corresponding analytical value, respectively.

centrations of INH and AcINH present in the sample. As is evident in Table 2, at a molar ratio of INH:AcINH greater than 4:1 the mean error in the recovery rate of AcINH exceeds 5%.

Although the data are not shown, we found that immediate cooling of samples on ice and early precipitation of protein was of crucial importance to the stability of both INH and AcINH, delay causing a marked decrease in concentrations. Although immediate analysis of samples is the ideal, concentrations of INH and AcINH in biological fluid samples remained constant for at least 3 h if immediately cooled on ice allowing adequate time for delivery and analysis. For reasons which are not clear, freezing of samples followed by thawing also caused a loss of analyte making long-term storage unsatisfactory.

4. Discussion

In the unmodified method of Lacroix et al. [4] 10 μl of 1% CA in methanol per 200 μl of analysate was used in the derivatization step. This concentration is not ideal since the yield of chromophoric derivative is highly sensitive to small changes in the CA content in this region as shown in Fig. 3. By increasing the CA content to 40 μl per 200 μl of analysate, maximal and stable concentrations of derivative could be achieved as shown both for free INH and for INH hydrolytically liberated from AcINH.

Although the hydrolytic yield of INH from AcINH is sub-maximal after 1 h of hydrolysis as allowed by Lacroix et al. [4]—para-ordinal dashed line in Fig. 4—it was clear from the quality of the calibration curves that extension of this time is neither necessary nor practical. It is apparent from the relevant curves, shown in Figs. 3 and 4, that the yield of INH from AcINH is less than that from matrix INH subjected to the same hydrolyzing conditions. It is reasonable to assume that the apparent discrepancy is due to hydrolytic degradation of a fraction of INH, and INH liberated from AcINH, to non-chromophoric products at 340 nm [6].

From Fig. 6 (graphs marked d and e), it is clear that Hz is stable under hydrolyzing conditions since the slopes of the calibration curves for Hz under hydrolyzing and non-hydrolyzing conditions coincide. The difference in the slopes of the calibration curves of MAcHz and DAcHz, graphs marked f and g in Fig. 6, is due either to a difference in the hydrolytic yield of Hz or to a difference in the rate of hydrolysis, or both. Consequently, it is not possible to quantitate MAcHz and DAcHz in patient samples.

Although the method is not ideal for the quantitation of low concentrations of AcINH in the presence of high concentrations of INH, it is satisfactory (intrasample concentration S.D. $<$ 5%) for the quantitation of AcINH in patient samples in the post-absorption phase using our formula which corrects for hydrolytic INH loss; 2 h after a high oral dose of INH (20 mg/kg) administered to a slow acetylator (data not shown) the ratio of AcINH:INH in plasma was greater than 1:4 and at no time were AcINH concentrations in urine lower than INH concentrations. The modified method described here has been used for the quantitation of INH, AcINH and Hz in patient samples as previously reported by us [7–9].

Acknowledgment

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