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DETERMINATION OF ISONIAZID METHANESULPHONATE AND ITS METABOLITES IN RABBIT BLOOD BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A rapid and sensitive high-performance liquid chromatographic method is described for simultaneous determination of isoniazid methanesulphonate (IHMS) and its metabolites, such as isoniazid (INH) and acetylisoniazid (AcINH) in rabbit blood. According to stability studies, IHMS was most stable at pH 3–5. After acidifying the blood to pH 5.0, a suitable amount of acetonitrile was added to the supernatant for extraction and niacinamide served as an internal standard. After evaporation, the residue was reconstituted with phosphate buffer and aliquots of this solution were separated on a reversed-phase phenyl column by a mobile phase consisting of 0.25 m*M* tetrabutylammonium phosphate as a paired-ion reagent. UV detection was performed at 280 nm. Under these conditions, the between-run coefficients of variation of IHMS, INH and AcINH from 1 to 25 μm/ml were 4.7 ± 2.5, 5.4 ± 1.0 and $5.1 \pm 3.1\%$, respectively. Hence this sensitive, reproducible and accurate method was suitable for pharmacokinetic studies of IHMS.

INTRODUCTION

Isoniazid (INH), which has remarkable antituberculosis activity, is widely used as the primary drug for phthisotherapeutics. However, some adverse reactions may occur during the INH treatment. Peripheral neuritis is a well known side-effect of INH which can be prevented by simultaneous administration of pyridoxine [1]. In addition to neuritis, there are many clinical reports indicating that significant hepatic damage may occur in 15–20% of patients and irreversible hepatic damage to fatality in up to 1% [2,3]. It is suggested that the hepatitis is mediated by the reactive metabolites hydrazine and acetylhy-

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drazine [4,5]. Owing to these adverse effects of INH, it is worth developing a less toxic derivative.

Isoniazid methanesulphonate (IHMS; isonicotinic acid 2-sulphomethylhydrazide) and isoniazid glucuronate are typical derivatives with a less toxic reaction and have equal antituberculosis activity in vivo and in vitro [6,7]. Our interest in IHMS was encouraged from a report by Orlowski et al. [7], that the toxicity of IHMS is only one seventh of that of INH and it can be used with higher doses to maintain continuously an antituberculosis concentration in blood. It may act as an ideal antituberculosis drug for more reasonable phthisic therapy.

Only one quantitative method has been documented for the determination of IHMS. In the Japan Pharmacopoeia VIII, an indirect titration method was applied to determine IHMS by hydrolysing it first with dilute hydrochloric acid to INH. However, this method can only detect milligram levels of IHMS and cannot be used to determine the concentration of IHMS at microgram levels in blood [8]. In addition, the strong acid hydrolysed not only IHMS but also the acetyl metabolite to INH in biological samples [9]. It is impossible to determine IHMS and its metabolites exactly and simultaneously with the above method.

In this paper, we describe an HPLC method that can directly detect IHMS and its metabolites INH and acetylisoniazid (AcINH) simultaneously and accurately in rabbit blood without any interferences. By means of this specific and sensitive method, the pharmacokinetics of IHMS can be studied.

EXPERIMENTAL

Materials

IHMS-Na corresponded to Japan Pharmacopoeia VIII grade (>99%). Analytical-reagent-grade INH (Wako, Osaka, Japan), niacinamide (Sigma, St. Louis, MO, U.S.A.), tetrabutylammonium phosphate (Waters Assoc., Milford, MA, U.S.A.) and other commercially available chemicals were used. AcINH was synthesized from INH according to the method developed by Fox and Gibas [10]. The melting point was 159–161 °C and the elemental analysis was C 53.69, H 5.01, N 23.50% (calculated: C 53.62, H 5.07, N 23.45%).

Apparatus

The liquid chromatograph consisted of an M-45 pump (Waters Assoc.), an M-7125 injector (Rheodyne), a 300 mm × 3.9 mm I.D. phenyl column (10 μm) (Waters Assoc.) and an M-45 UV detector (Waters Assoc.). The mobile phase was a 10 m*M* phosphate buffer containing 0.25 m*M* tetrabutylammonium phosphate as paired-ion source (pH 4.1) and the flow-rate was 1.0 ml/min. The chromatograph was operated at ambient temperature and the wavelength of the detector was set at 280 nm.

Sample preparation

To 1 ml of rabbit blood, 0.6 g of ammonium sulphate and 140 μl of 0.2 *M* phosphoric acid solution containing an internal standard (niacinamide) were added immediately. Following gentle shaking, the mixture was centrifuged at 10 000 *g* for 10 min and the supernatant was mixed with 1 ml of acetonitrile three times. After extraction, the organic layer was transferred into a glass tube and evaporated to dryness under nitrogen. Finally, the residue was dissolved in 100 μl of phosphate buffer (pH 4.1) and aliquots of this solution were injected into the HPLC system.

Calibration graphs

Calibration graphs were obtained by adding IHMS-Na, INH and AcINH to rabbit blood at concentrations of 1–25 μg/ml.

Stability studies

In 0.1 ionic strength buffer solution. Buffer solutions of pH 2.6, 3.0, 4.3, 5.1, 6.6, 7.3, 8.6 and 9.6 with an ionic strength of 0.1 were prepared according to the Lewis method [11,12]. To each buffer solution, IHMS-Na was added to make the solution 0.1 m*M* and then incubated at 25 ± 0.5 °C. After a suitable time, 1 ml of solution was removed and, after adding niacinamide as an internal standard, 50 μl of the sample solution was injected immediately into the HPLC system.

In rabbit blood. To 10 ml of rabbit blood of pH 7.4 (blank) and pH 5.0 (adjusted with 0.2 *M* phosphoric acid), IHMS-Na was added to make the blood solution 0.1 m*M* and then incubated at 25 ± 0.5 °C. Subsequently, 200 μl of blood were taken after an adequate time, then 0.3 g of ammonium sulphate and phosphate solution containing niacinamide were added immediately to make the final pH 5.0. After gentle shaking, the mixture was centrifuged at 10 000 *g* for 10 min and 50 μl of the supernatant were injected directly into the HPLC system.

Animal studies

A 55.4 mg/kg amount of IHMS-Na was dissolved in 2 ml of normal saline solution (pH 5.0) and injected intraperitoneally into healthy male rabbits. Blood samples were collected from the marginal vein at appropriate times and 1 ml of blood was used for analysis.

The elimination rate constant of IHMS-Na was determined using the first-order kinetic theory.

The data were expressed as means ± S.D. and Student's *t*-test was used for statistical analysis.

RESULTS AND DISCUSSION

Owing to the acid-labile property described previously [8], the stability of IHMS at various pH should be determined first. As shown in Fig. 1, IHMS was stable in buffer solution from pH 2.6 to 5.0. The range of the degradation half-life for IHMS was from 4.4 ± 1.7 to 2.7 ± 1.1 days. However, the degradation rate of IHMS increased together with the pH value above 5.0 ($y = -6.13 + 0.47x$, $r = 0.995$). This result indicates that IHMS might be unstable in the physiological state of blood. As shown in Fig. 2, the rate of degradation of IHMS in normal rabbit blood was very fast, and only 20% of IHMS could be detected after incubation for 25 min at room temperature. The degradation half-life of

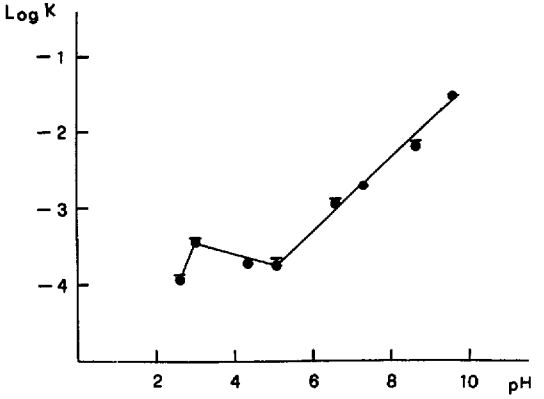

Fig. 1. pH dependence of degradation rate of IHMS-Na at 25°C. Concentration, 0.1 m*M*; ionic strength, 0.1. $n = 3$.

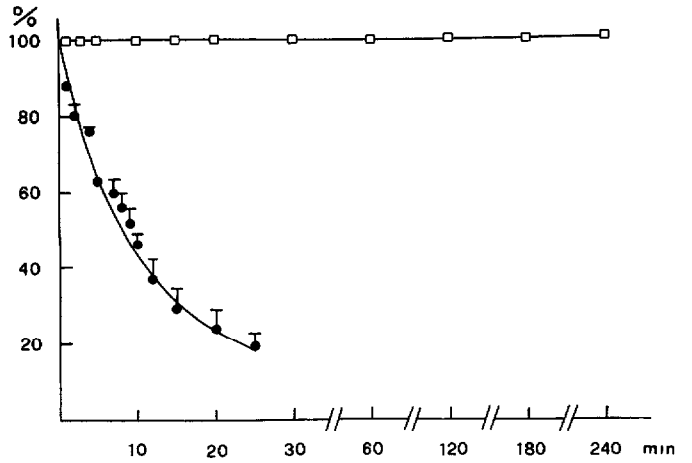

Fig. 2. Degradation of IHMS-Na in rabbit blood at pH 7.4 (●) and pH 5.0 (□). Concentration, 0.1 m*M*; temperature, 25°C. $n = 3$.

IHMS was 10.3 ± 2.8 min. On the other hand, if whole blood was adjusted to pH 5.0, no degradation of IHMS could be observed up to 4 h. Therefore, the sampled blood should be adjusted to pH 5.0 immediately and then none of the IHMS will be degraded during the analytical process.

Extraction was useful for avoiding interference from biological substances in HPLC analysis. For efficient extraction, ammonium sulphate was added to precipitate protein and to salt out desired compounds. Acetonitrile was used as the extraction solvent. With this method, the recoveries of IHMS, INH and AcINH within the concentration range 1–25 μg/ml at pH 3.0, 4.0 and 5.0 are listed in Table I. As shown, both IHMS and INH gave the best recoveries at pH 5.0, but there was no obvious statistical difference for AcINH ($p > 0.05$). It appeared that the blood at pH 5.0 not only stabilized IHMS in the blood but also gave the best recovery.

Owing to the high polarity of IHMS, the capacity factor of IHMS in a reversed-phase column was less than 0.5, which resulted in blood component peaks interfering with the IHMS peak. Tetrabutylammonium phosphate, which was added to the mobile phase (0.25 m*M*) as a paired-ion reagent, not only increased the capacity factor of IHMS but also separated IHMS from interfering blood components.

Following the above procedure, all compounds were resolved within 20 min without interference (Fig. 3). The retention times were 8.3 ± 0.1, 9.3 ± 0.1, 12.4 ± 0.2 and 17.8 ± 0.2 min for AcINH, INH, niacinamide and IHMS, respectively ($n = 4$). The coefficients of variation of the retention time were all within 2% and showed satisfactory reproducibility. The accuracy of the method is shown in Table II. The between-run calibration graphs ($n = 4$) were linear with regression coefficients greater than 0.999. The coefficients of variation for IHMS, INH and AcINH were all within 10%. As the therapeutic concentration of INH in serum is 1–3 μg/ml [13], this method is applicable to the determination of antituberculosis concentrations of IHMS in vivo.

The blood concentrations of IHMS, INH and AcINH after intraperitoneal administration of IHMS-Na (55.4 mg/kg) to six rabbits are shown in Fig. 4.

TABLE I

RECOVERIES OF IHMS, INH AND AcINH FROM RABBIT BLOOD UNDER DIFFERENT pH CONDITIONS

pH	Recovery (mean ± S.D., $n = 5$) (%)		
	IHMS	INH	AcINH
3.0	59.5 ± 5.2	88.4 ± 4.1	104.2 ± 5.6
4.0	66.2 ± 7.7	91.0 ± 10.1	100.8 ± 11.6
5.0	71.3 ± 5.5	97.6 ± 2.7	104.6 ± 3.4

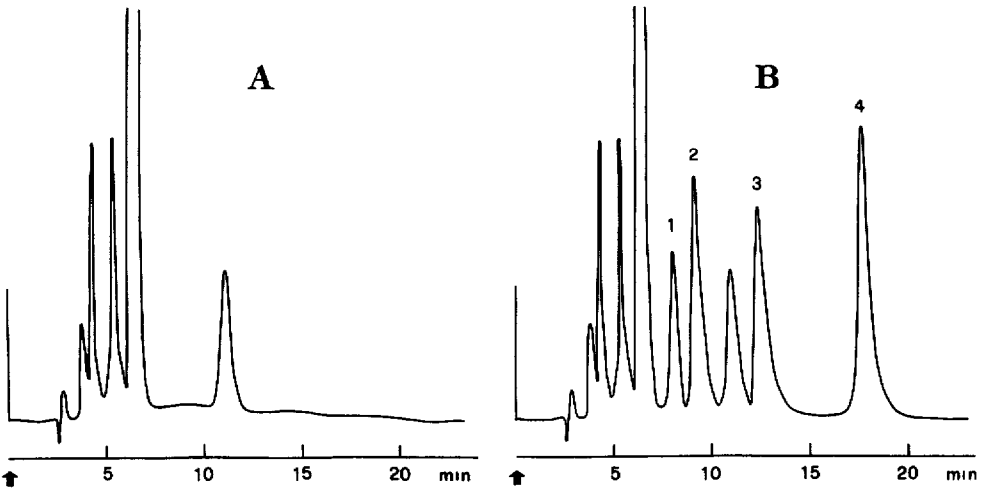

Fig. 3. HPLC of IHMS and its metabolites INH and AcINH extracted from rabbit blood. (A) Blank; (B) after administration of IHMS-Na. Peaks: 1 = AcINH; 2 = INH; 3 = niacinamide (internal standard); 4 = IHMS.

TABLE II

ACCURACY OF DETERMINATION OF IHMS, INH AND AcINH (BETWEEN RUNS)

Correlation coefficient > 0.999, $n = 4$.

Compound	Concentration (μg/ml)	Linear regression	Standard error of curve	C.V. (mean ± S.D.) (%)
IHMS	1-25	$y = 0.658 + 19.6x$	±0.331	4.72 ± 2.46
INH	1-25	$y = 0.889 + 15.2x$	±0.473	5.35 ± 0.95
AcINH	1-25	$y = -0.290 + 9.5x$	±0.100	5.13 ± 3.10

Applying the one-compartment open-body model with degradation of IHMS at the absorption site [14–16], the pharmacokinetic parameters of IHMS and its metabolites in rabbits were calculated and are listed in Table III. Absorption and elimination of IHMS after intraperitoneal administration of IHMS-Na were very fast. The peak concentration of IHMS appeared 10 min after administration and quickly decayed within 2 h. As IHMS decayed, INH and AcINH increased gradually and lasted up to 7 h. The absolute bioavailability of IHMS was 0.63 ± 0.19 (by comparing the respective AUCs of IHMS after intraperitoneal and intravenous administration of IHMS-Na at same dosage level) [16]. The results showed that IHMS was rapidly eliminated and INH and AcINH were formed simultaneously. Therefore, the antituberculosis activity of IHMS might come mainly from its active metabolite, INH.

As indicated above, the method described can determine IHMS and its me-

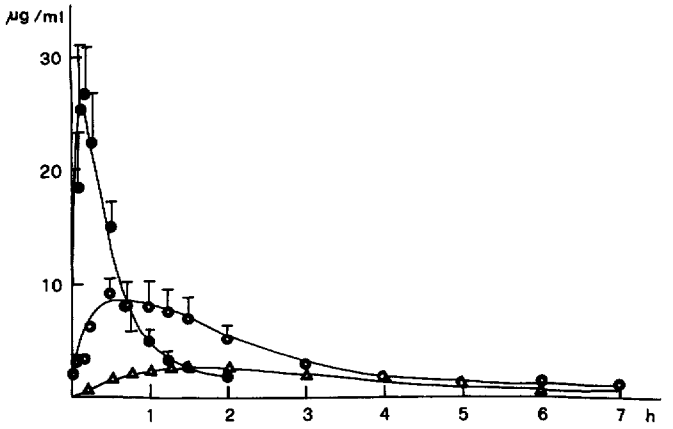

Fig. 4. Blood concentration of (●) IHMS, (○) INH and (△) AcINH after intraperitoneal administration of IHMS-Na (55.4 mg/kg) to rabbits. $n=6$.

TABLE III

PHARMACOKINETIC PARAMETERS OBTAINED AFTER INTRAPERITONEAL ADMINISTRATION OF IHMS-Na (55.4 mg/kg) TO RABBITS

Results are given as mean ± S.D. ($n=6$).

Parameter	Value
Absorption rate constant of IHMS (k_a)	18.0 ± 9.1 h^{-1}
Half-life of IHMS ($t_{\frac{1}{2}}$)	0.367 ± 0.117 h
Apparent distribution volume of IHMS (V)	1.96 ± 0.96 l/kg
Systemic clearance of IHMS (Cl)	3.75 ± 1.39 l/h·kg
Degradation rate constant of IHMS to INH at the absorption site (k_{02})	4.26 ± 4.03 h^{-1}
Elimination rate constant of IHMS (k_{10})	0.928 ± 0.397 h^{-1}
Formation rate constant of INH from IHMS (k_{12})	1.12 ± 0.40 h^{-1}
Elimination rate constant for INH (k_{20})	0.464 ± 0.185 h^{-1}
Area under the blood concentration–time curve (AUC):	
$AUC_{0-\infty}$ IHMS	17.6 ± 6.4 μg/ml·h
$AUC_{0-\infty}$ INH	24.7 ± 9.9 μg/ml·h

tabolites directly in rabbit blood with high sensitivity, selectivity and accuracy, and is suitable for pharmacokinetic studies of IHMS [16].

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