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Short Communication

Simultaneous assay for isoniazid and hydrazine metabolite in plasma and cerebrospinal fluid in the rabbit

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ABSTRACT

A simple procedure for the simultaneous determination of isoniazid and hydrazine metabolite in plasma and cerebrospinal fluid in the rabbit is described. The assay involves organic extraction before and after derivatization of the two compounds and the internal standard, phenelzine. The extract of the derivatized compounds was evaporated to dryness at 40°C and the residue redissolved in the mobile phase (50 μl). A 25-μl aliquot was injected into the liquid chromatograph and eluted with acetonitrile–water–triethylamine (70:30:0.4, v/v) containing 5 m*M* heptanesulphonic acid on a 30-μm C_8 precolumn linked to a 10-μm C_{18} μBondapak column at ambient temperature (25 ± 1°C). The eluate was detected by ultraviolet detection at 320 nm.

INTRODUCTION

Many procedures have been described for the assay of isoniazid (INH) in plasma [1–6]. The fluorometric assay [1], although sensitive, is laborious and expensive. The hydrazine (HYD) metabolite was poorly extracted (1%) and did not show fluorescence. Several procedures based on high-performance liquid chromatography (HPLC) for assay of INH with or without metabolites have been published [3–6]. In some [3,4], a large sample volume and drastic chromatographic conditions were used, and in the others [5,6] the supernatant injected would endanger the chromatographic system and did not include an internal standard, thus making the results questionable.

HYD is a potent toxin and carcinogen [7]. It is formed during the direct conversion of INH into isonicotinic acid. In the wake of the proposal that monoacetylhydrazine plays a role in INH-related toxicity, HYD has been implicated as a possible culprit [8]. There is apparently no well established procedure for direct estimation of total HYD formed during INH metabolism. Gas chromatography–electron-impact mass spectrometry [9] and the indirect estimation of total HYD

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by determining the ratio of INH metabolized in the major pathways [8] are laborious and expensive.

This paper describes the development of a simultaneous assay for INH and HYD, to examine the relationship of the concentrations of the two compounds. The procedure of organic solvent extraction before and after derivatization and concentration of the solvent extract was adopted to optimize sensitivity and selectivity. The chromatographic system used was a modification of that used by other workers [10,11].

EXPERIMENTAL

Reagents

Salicylaldehyde, triethylamine and hydrazine hydrate (24% in water) (Merck, Darmstadt, Germany) were of analytical grade (AR). INH and phenelzine (PHEN) sulphate were purchased from Universal Pharmaceuticals Labs. (Hong Kong). Other reagents used were: heptanesulphonic acid (Sigma, St. Louis, MO, U.S.A.), acetic acid and acetonitrile (AR grade, Merck).

Apparatus and chromatographic conditions

The chromatographic system consisted of a Waters M45 pump (Waters Assoc., Milford, MA, U.S.A.), a Model 7125 injector (Rheodyne, CA, U.S.A.) and a variable-wavelength Hitachi 220-S UV detector with a built-in chart recorder (Hitachi, Tokyo, Japan). Analyses were performed on a μBondapak reversed-phase C_{18} column (10 μm, 300 mm × 3.9 I.D., Waters Assoc.) linked to a C_8 precolumn (30 μm, 30 mm × 4.6 mm I.D., Waters Assoc). The mobile phase was acetonitrile–water–triethylamine (70:30:0.4, v/v) with 5 m*M* heptanesulphonic acid at pH 6.0 (adjusted with acetic acid). Other conditions, flow-rate 1 ml/min; pressure, 35 bar; ambient temperature, 25± 1°C; UV detection wavelength, 320 nm. All glassware was treated as described previously [12], to reduce loss of drugs by adsorption on the glass walls.

Synthesis of reference compounds

The salicylhydrazones of isoniazid (INH-HDZ) and phenelzine (PHEN-HDZ) and the salicylazine of hydrazine were synthesized by a modification of the procedure used by Jenner and Ellard [10]. A standard amount of INH or phenelzine sulphate or hydrazine hydrate was shaken with excess salicylaldehyde in ethanol, and the mixture was heated in a water-bath at 60°C for 30 min. The products were purified by crystallization in ethyl acetate and were distinguished from the parent compounds by their UV absorption spectra and by thin-layer chromatography (TLC: system T1) [13]. Briefly, the compounds, in methanolic solutions of 1 mg/ml, were spotted on a silica plate (coated with silica G 60, 0.2 mm thick; Sigma) 1.5 cm from the bottom line. The plate was placed in a tank containing ammonia–methanol (1.5:100) to a depth of 0.5–1 cm, and the tank was made

airtight with a lid. After 30 min the plate was dried in air and the compounds were located by potassium iodoplatinate spray (Sigma).

Sample preparation

Samples of rabbit plasma and cerebrospinal fluid (CSF) were obtained as described earlier [14]. Plasma or CSF (0.2–0.5 ml) was pippetted into a 10-ml test-tube, and 50 μl of phenelzine sulphate (50 μg/ml) were added as internal standard. After the addition of 0.1 ml of aqueous acetic acid (v/v) to adjust pH to 4, the impurities were extracted by shaking with 5 ml of *n*-hexane for 30 min, and then centrifuged for 10 min at 1870 *g*. The organic layer was discarded. The sample was derivatized by addition of 0.3 ml of an ethanolic solution of 0.1% (v/v) salicylaldehyde and 0.4 ml of 10% aqueous acetic acid, and heated in a water-bath for 30 min at 60°C. After cooling to room temperature (25 ± 1°C) the sample was shaken for 10 s with 1 ml of 1 *M* K_2HPO_4 buffer (pH 6.5) for optimum extraction and removal of the excess salicylaldehyde. The salicylhydrazones and azine were extracted from the cooled aqueous mixture by shaking with 5 ml of diethyl ether for 10 min and centrifuging for 10 min at 1870 *g*. The extract was transferred into a tapered test-tube and evaporated dry under a stream of nitrogen at 40°C. The residue was dissolved in 50 μl of mobile phase, and duplicate 25-μl aliquots were injected into the HPLC system. The extraction and derivatization procedure took *ca.* 2 h for a batch of ten samples, and the derivatives could be stored for three days at 4°C without loss of accuracy.

RESULTS AND DISCUSSION

Characterization of the reference compounds

The derivatives absorbed UV radiation much more than the parent compounds, making the choice of detector appropriate. They exhibited larger retention factors because they are less polar. *viz.*: INH = 0.52 and INH-HDZ = 0.66; PHEN = 0.47 and PHEN-HDZ = 0.73; HYD = 0.28 and azine = 0.70; salicylaldehyde = 0.5. By dissolving appropriate amounts of these compounds in the mobile phase, the HPLC separation conditions were developed.

Derivatizing conditions of the sample

Heating of the reaction mixture for 30 min at 60, 80 and 90°C showed that the derivatives are thermolabile. The yield was poor at lower temperatures, and variable when the mixture was heated for a shorter time. The optimal reaction conditions were 60°C for 30 min. PHEN exhibited many intermediate products when heated for a shorter time. It was used as internal standard because of its similar structural and solubility properties to isoniazid. INH and PHEN are hydrazides and react with salicylaldehyde at the hydrazine functional group.

Performance of the HPLC system

Representative chromatograms of the compounds in standard solution, and of the plasma extract from a treated rabbit, are shown in Fig. 1. The analytical peaks are symmetrical, with retention times of 96 s for INH-HDZ, 180 s for PHEN-HDZ, and 240 s for the azine. The excess salicylaldehyde was resolved at 126 s. The peak-height ratio was satisfactory for quantitation to be performed. The run time for the assay was 9 min for plasma and 7 min for CSF.

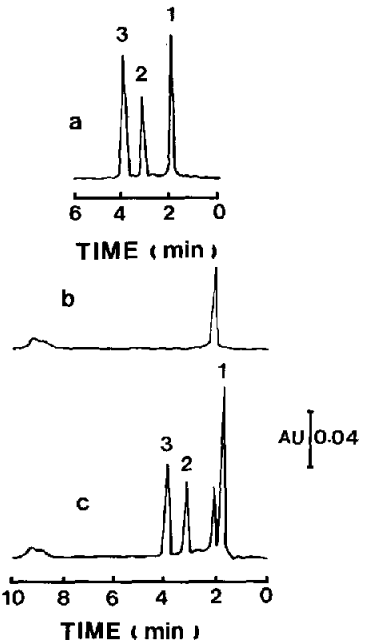

Fig. 1. Representative chromatograms of (a) a standard solution with concentrations equivalent to 0.5 μg/ml for INH and PHEN and 0.05 μg/ml for HYD, (b) drug-free plasma and (c) a plasma extract from a rabbit 5 h after an intravenous dose of INH (5 mg/kg). Peaks: 1 = INH; 2 = PHEN (internal standard); 3 = HYD.

Linearity, sensitivity, precision, recovery and selectivity

Concentration ranges of 0.5–10 μg/ml for INH and 76.8–768.0 ng/ml for HYD, in plasma or CSF, were used for standardization and regression analysis. The mean correlation coefficients for six standardization curves were 0.993 for INH and 0.9997 for HYD. The standard curves for plasma and CSF were identical. The limit of detection was 0.2 μg/ml for INH and 10 ng/ml for HYD. To assess recovery, peak heights of the directly injected INH-HDZ or azine were compared with those obtained after applying the extraction procedure to INH-HDZ or azine, and with INH or HYD when taken through the whole procedure

of derivatization and extraction at concentrations of 0.5 and 5 μg/ml for INH and 153.6 ng/ml for HYD ($n=5$). The efficiency of derivatization was 88% at 0.5 μg/ml and 83.6% at 5 μg/ml for INH and 73.7% at 153.6 ng/ml for HYD. Extraction of the hydrazones was 59.5% at 0.5 μg/ml and 60.2% at 5 μg/ml for INH, and 67.4% at 153.6 ng/ml for HYD; this gave an overall recovery of 52.4% at 0.5 μg/ml and 50.3% at 5 μg/ml for INH, and 49.7% at 153.6 ng/ml for HYD. Drugs such as rifampicin, pyrazinamide and *p*-aminosalicylic acid were tested, and did not interfere with the assay. Most of these are extracted in hexane and they do not react with the salicylaldehyde.

CONCLUSION

The procedure is economical and convenient with respect to the materials and sample volume used. Like other procedures [1,10], this one can only detect the acid-labile INH and metabolite. Monoacetylhydrazine was not determined under the conditions described.

The assay allows direct determination of total HYD formed during INH metabolism. We have used the procedure to study the disposition and kinetics of INH and HYD in rabbits [15] and in elderly tuberculosis patients. The results and conclusions were similar to those obtained by other methods for rabbits [16] and humans [17]. The assay is a useful research and clinical tool in the study of HYD as a metabolite of INH.

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