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SEPARATION AND QUANTITATION OF HYDRALAZINE METABOLITES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

A selective high-performance liquid chromatographic assay for the separation and quantitation of the proposed hepatic microsomal metabolites of hydralazine (HP), phthalazine, phthalazinone, *s*-triazolo[3,4-*a*]phthalazine, 3-methyl-*s*-triazolo[3,4-*a*]phthalazine and 3-hydroxymethyl-*s*-triazolo[3,4-*a*]phthalazine, is described. An extraction technique was developed for the removal of HP from hepatic microsomal samples in order to minimize the measurement of products resulting from the chemical degradation of HP. The effects of pH and composition of the mobile phase on the retention times and resolution of the five compounds were examined. The methods presented are accurate and reliable, permitting the baseline separation of five HP metabolites with reasonable analysis time and sensitivity.

INTRODUCTION

Hydralazine (HP) is an antihypertensive agent which is widely used in combination with a β-blocking drug and a diuretic for the treatment of essential

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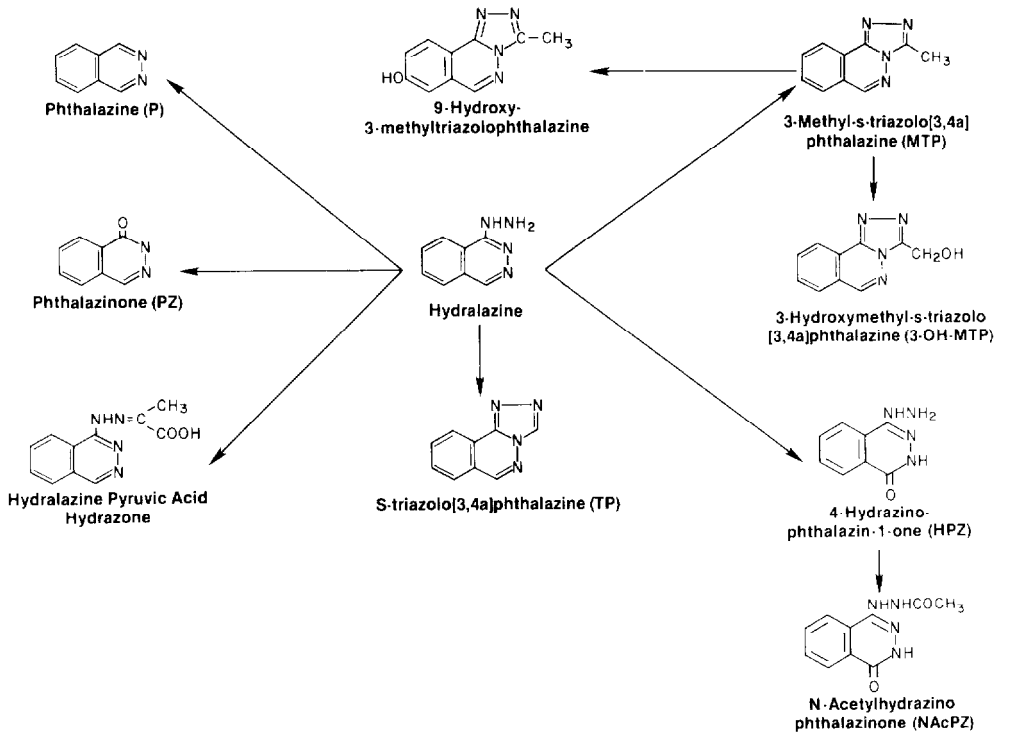


Fig. 1. Proposed pathways for the metabolism of hydralazine.

hypertension [1, 2]. The metabolism of HP in man and experimental animals has been the subject of several investigations [3–7]. HP is extensively metabolized to products that are excreted predominantly in the urine. It has been shown that the drug undergoes N-acetylation, oxidation, hydroxylation, hydrazone formation and conjugation (Fig. 1). Studies on the metabolism of HP have been prompted in part by the possible involvement of metabolites in the side-effects of the drug. The chronic use of HP is associated with the development of a syndrome resembling systemic lupus erythematosus [8]. HP-induced lupus occurs primarily in phenotypically slow acetylators of the drug, suggesting that the metabolism of HP may play a role in the development of adverse effects [9].

The identification and quantitation of HP and its metabolites in plasma and urine have always presented problems because HP is unstable at high pH and cannot be extracted from biological fluids at low pH [10–12]. In order to circumvent these difficulties, investigators have derivatized HP prior to extraction. A problem with most of these assays is that the acid-labile hydrazone metabolites of HP are reconverted to HP by the acidic conditions of the derivatization procedures [13]. The reported values for HP obtained with such assays were found to be incorrectly high [13]. In addition, the regenerated HP can react with various biogenic aldehydes and ketones, such as pyruvic acid, which are present in both plasma and urine [14], or undergo chemical degradation processes, resulting in the formation of metabolites of HP. Therefore, most of the available assay methods, including those employing spectro-

photometry, gas chromatography (GC) or high-performance liquid chromatography (HPLC), have been found to lack the selectivity to distinguish between HP and its metabolites in biological fluids. Since the toxicity of HP appears to be related to its metabolism, it is important to have a selective method for the measurement of the metabolic products of HP in various tissues. For this reason, an HPLC assay has been developed for the identification and quantitation of the major HP metabolites. The data presented in this communication demonstrate the suitability of the assay for the evaluation of the metabolism of HP by rat liver microsomes.

EXPERIMENTAL

Materials

Hydralazine hydrochloride was supplied by Ciba-Geigy (Summit, NJ, U.S.A.). Phthalazine (P) and phenacetin were obtained from Aldrich (Milwaukee, WI, U.S.A.) and phthalazinone (PZ) was obtained from Pfaltz and Bauer Research Chemicals (Stamford, CN, U.S.A.). *s*-Triazolo[3,4-*a*]-phthalazine (TP), 3-methyl-*s*-triazolo[3,4-*a*] phthalazine (MTP) and 3-hydroxy-MTP (3-OH-MTP) were prepared by previously published procedures [15- 17]. The purity of all compounds was confirmed by HPLC. Nicotinamide-adenine dinucleotide phosphate (NADPH) was purchased from Sigma (St. Louis, MO, U.S.A.). Reagent-grade inorganic materials and HPLC-grade organic solvents were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.).

High-performance liquid chromatography

A Waters HPLC system was used which included a Waters Intelligent sample processor (WISP) Model 710A for automatic sample injection. A Waters Model 6000A pump delivered the mobile phase to a Waters μBondapak (10 μm particle size) C_{18} reversed-phase column (30 cm × 3.9 mm I.D.) at a flow-rate of 1.5 ml/min. The mobile phase was 0.01 *M* sodium acetate buffer—methanol (92:8), pH 2.75. The eluent was monitored by absorbance at 254 nm with a Waters Model 441 UV detector (Waters Assoc., Milford, MA, U.S.A.). Detector sensitivity was 0.5 a.u.f.s. A Hewlett-Packard 3390A programmable integrator was interfaced with the UV detector to record and analyze the data (Hewlett-Packard, Avondale, PA, U.S.A.).

Sample preparation

Adult, male Sprague–Dawley rats (200–224 g) were obtained from Hilltop Laboratory Animals (Scottdale, PA, U.S.A.) and used in all experiments. Animals were maintained under standardized conditions of light (06:00 a.m.–06:00 p.m.) and temperature (22°C). Food (Wayne Laboratory Chow) and water were provided ad libitum. Animals were killed by decapitation and livers were immediately removed and placed in cold 1.15% KCl- 50 m*M* Tris buffer (pH 7.4). Tissues were homogenized and microsomes were obtained by differential centrifugation as previously described [18]. Microsomal pellets were resuspended in KCl—Tris buffer at a concentration equivalent to 125 mg tissue per ml.

Stock solutions of P, PZ, TP, MTP and 3-OH-MTP standards were

prepared in HPLC-grade methanol. Rat hepatic microsomes (1 ml) and 1 ml of 3 m*M* $MgCl_2$—50 m*M* Tris buffer (pH 7.4) were placed in 25-ml Erlenmeyer flasks and kept on ice throughout the procedure. The contents of the flasks were spiked with six concentrations of metabolite standards: 0.47, 0.94, 1.88, 3.75, 7.5 and 15.0 μg/ml. The standards were prepared so that each concentration was delivered in a volume of 50 μl to the sample flasks. The internal standard, phenacetin, was prepared so that a volume of 125 μl would deliver 0.625 μg/ml.

For studies on the microsomal metabolism of HP, incubation media contained 0.05 *M* Tris—HCl (pH 7.4), 5.0 m*M* $MgCl_2$ and microsomal protein (4 mg protein per ml) in a total volume of 2.0 ml. Where indicated, NADPH (0.5 m*M*) was included in the incubation medium. Incubations were done in 25-ml Erlenmeyer flasks at 37°C under air in a Dubnoff metabolic incubator. The reaction was initiated by the addition of HP (4 m*M*) in 50 μl of $MgCl_2$—Tris buffer. After incubation, all flasks received 80 ng of phenacetin to serve as an internal standard. One set of flasks, which received the internal standard but was not incubated, served as the zero-time control. Additional control flasks included microsomal preparations incubated without HP and HP incubated in buffer but without microsomes. Incubations were terminated by the addition of ice-cold methylene chloride (5 ml).

Extraction procedure

The contents of the flasks were extracted with 5 ml of methylene chloride. A 3-ml volume of the organic layer was filtered through a 0.45-μm Metricel membrane filter (Gelman Science, Ann Arbor, MI, U.S.A.) into disposable glass culture tubes and evaporated to dryness under a stream of air at room temperature using a Meyer N-Evap analytical evaporator (Organomation Assoc., South Berlin, MA, U.S.A.). The data obtained from samples evaporated under nitrogen were not different from those obtained when air was used. The residue was redissolved with 200 μl of a 30% methanol—water solution and 75 μl were injected into the HPLC system. Extraction efficiencies were determined by spiking microsomal suspensions with various amounts of the standards and carrying them through the procedure described above. The recovery of each compound was determined by comparing the peak heights of extracted standards with those of unextracted (external) standards. The external standards were prepared by the addition of stock solutions of P, PZ, TP, MTP, and 3-OH-MTP to a 30% methanol—water solution to give concentrations equivalent to that of extracted standards.

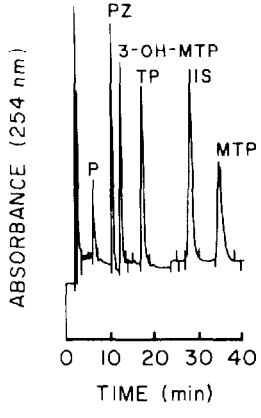
Quantitation

The standard curves were constructed by plotting the peak-height ratios of each metabolite relative to the internal standard versus the concentration of metabolite (0.47—15.0 μg/ml). The linearity of each curve was determined by unweighted linear regression analyses. The intra-assay reproducibilities were determined by assaying four replicate microsomal samples containing added amounts of metabolites at concentrations ranging from 0.47 to 15.0 μg/ml.

RESULTS AND DISCUSSION

Fig. 2 is a representative chromatogram illustrating the separation of P, PZ, TP, MTP, 3-OH-MTP and the internal standard, phenacetin, using the HPLC method described. In the development of this analytical procedure, the effects of mobile phase composition on the retention times of the five HP metabolites were investigated. The retention times of the metabolite standards decreased with increasing concentrations of methanol in water (pH 2.75; Fig. 3). Fig. 4 illustrates the effect of water versus buffer (pH 2.75) in 12% methanol on the resolution of HP metabolite standards. When sodium acetate buffer was used as the aqueous component of the mobile phase, resolution of all five compounds was obtained and the retention times of all metabolites were shorter than when water was the aqueous component. This effect may be due to increased solubility of the metabolites in buffer, causing a decrease in retention on the column. The metabolites interact with the ions in the buffer, resulting in a caging effect which decreases interaction with the column packing material and reduces band tailing. The overall effect of the buffer is to yield symmetrical peaks and optimal resolution with shorter retention times.

The effects of pH on the retention times and resolution of the five compounds were investigated using a mobile phase of 8% methanol in 0.01 *M* sodium acetate buffer. The pH was varied by using different amounts of acetic acid. Fig. 5 illustrates the dramatic effects of pH on the retention times and resolution of all of the metabolite standards. This may be due to the ionization of the compounds resulting in increased solubility in the mobile phase and



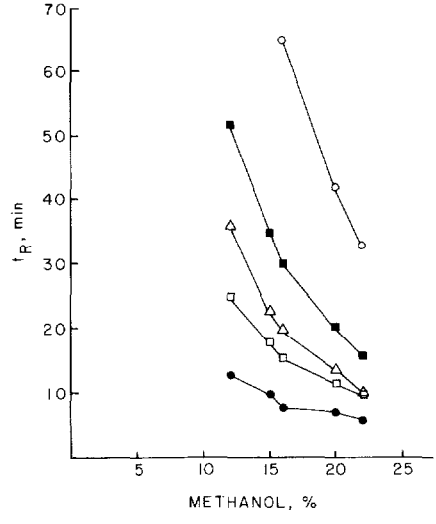


Fig. 2. HPLC profile of P, PZ, TP, MTP, 3-OH-MTP and the internal standard (IS), phenacetin. HP metabolite standards and phenacetin were separated using a C_{18} reversed-phase column and a mobile phase consisting of 8% methanol—0.01 *M* sodium acetate buffer (pH 2.75) and the eluent was monitored by UV absorbance at 254 nm, as described in Experimental.

Fig. 3. Effect of percentage methanol in water (pH 2.75) on the retention times (t_R) of P (●), PZ (□), TP (■), MTP (○) and 3-OH-MTP (△).

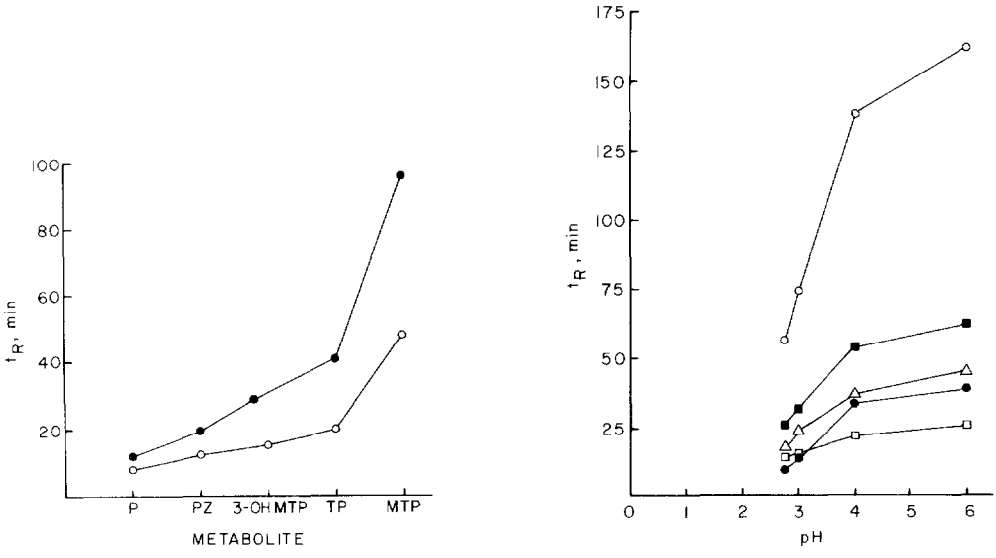


Fig. 4. Effect of 0.01 *M* sodium acetate buffer (○), pH 2.75, versus water (●), pH 2.75, in 12% methanol on the retention times (t_R) of P, PZ, TP, MTP and 3-OH-MTP.

Fig. 5. Effect of pH of the mobile phase (8% methanol—0.01 *M* sodium acetate buffer) on the retention times (t_R) of P (●), PZ (□), TP (■), MTP (○) and 3-OH-MTP (△).

therefore decreased retention on the column. Optimal resolution and retention times were achieved at pH 2.75.

Because our primary objective was to develop a method to study the hepatic microsomal metabolites of HP, we used an extraction technique which removed HP from the samples, thereby minimizing the measurement of products resulting from the chemical degradation of HP. This approach also avoided the inherent problems involved with the derivatization of HP. HP was removed from the samples by decreasing the pH of the incubation medium. The pK_a of HP is approx. 7.0. When the internal standard was added in 125 μl of glacial acetic acid, the pH of the incubation medium was decreased to approx. 3.0. At this pH, HP is completely ionized and therefore more water-soluble. Upon extraction with an organic solvent, HP remained in the aqueous layer. When the internal standard was added in water or methanol, which has little effect on the pH of the medium, HP was extracted into the organic layer. Under the latter conditions, the formation of chemical degradation products of HP, including P and PZ, complicated interpretation of experimental data (data not shown). The procedure for the removal of HP from the incubation medium takes approx. 10 min, and during this interval, HP is susceptible to chemical degradation. However, since HP is more stable under the acidic conditions used than at neutral pH [12], only minimal quantities of degradation products (P and PZ) are formed. The specificity of the assay is not compromised by the extraction procedure.

In the development of an extraction method, several organic solvents were used, including diethyl ether, benzene, benzene–heptane (1:1) and methylene chloride. The solvent found to have the best extraction efficiency for all five metabolites of HP was methylene chloride. The mean (± S.E.) recoveries (%)

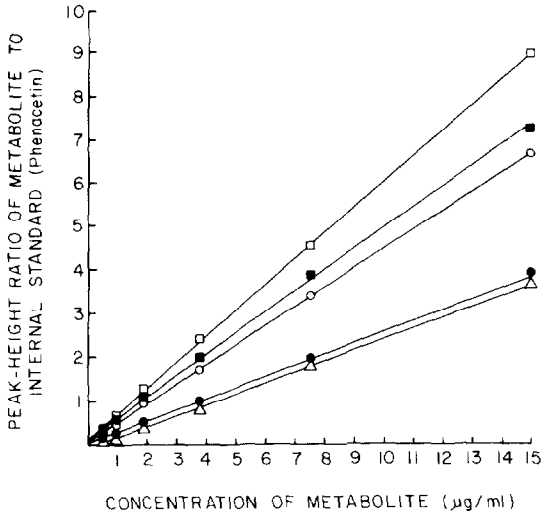


Fig. 6. Standard curves for the HPLC analyses of P (△), PZ (□), TP (○), MTP (●) and 3-OH-MTP (■) in rat liver microsomes with correlation coefficients of 0.9988, 0.9993, 0.9998, 0.9999 and 0.9963, respectively. Extraction and HPLC conditions were as described in Experimental. Each point represents the mean value of four determinations and the standard error values are all less than 5% of the means.

TABLE I

INTRA-ASSAY REPRODUCIBILITY FOR THE MEASUREMENT OF HYDRALAZINE METABOLITES

Intra-assay coefficients of variation were determined over the concentration range 0.47—15.0 μg/ml; four replicate determinations at each concentration.

Metabolite	Coefficient of variation (%)					
	15 μg/ml	7.5 μg/ml	3.75 μg/ml	1.88 μg/ml	0.94 μg/ml	0.47 μg/ml
P	8.2	8.5	7.2	12.0	7.4	7.1
PZ	20.4	2.0	2.7	2.2	1.0	4.0
3-OH-MTP	10.3	3.1	1.0	0.7	5.4	2.6
TP	0.9	2.0	3.2	0.7	1.5	5.8
MTP	9.0	1.4	2.0	1.3	2.6	8.0

for the 3.75 μg/ml concentrations of P, PZ, 3-OH-MTP, TP and MTP were 81.0 ± 3.0, 93.8 ± 0.5, 76.0 ± 1.0, 94.5 ± 1.5 and 98.5 ± 0.5, respectively. At the 0.47 μg/ml concentrations, the mean (± S.E.) recoveries (%) were 67.5 ± 0.5, 90.8 ± 5.4, 72.8 ± 1.8, 89.5 ± 4.5 and 85.5 ± 5.7. The standard curves, obtained by spiking microsomal supsensions with various concentrations of the metabolite standards, are shown in Fig. 6. The curves were linear ($r > 0.996$) over the concentration range 0.47—15.0 μg/ml. The intra-assay coefficients of variation for the assay ranged from 1 to 20% over the concentration range 0.47—15.0 μg/ml (Table I). The limits of detection (determined by peak height twice noise) were 0.3, 0.07, 0.08, 0.1 and 0.2 μg/ml for P, PZ, 3-OH-MTP, TP and MTP, respectively.

The simultaneous quantitation of various HP metabolites provided several

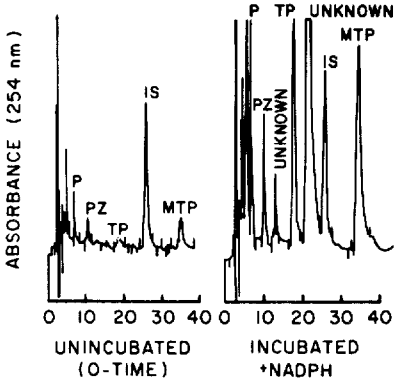


Fig. 7. Metabolism of hydralazine by hepatic microsomes in the presence of NADPH. Liver microsomes (4 mg protein per ml) were incubated with HP (4 m*M*) + NADPH for 10 min at 37° C as described in Experimental. After incubation, the internal standard (IS), phenacetin, was added to each flask and the samples were processed for HPLC analyses.

challenges in the development of both extraction and chromatographic methods. The methods presented are accurate and reliable, permitting the baseline separation of five HP metabolites with reasonable analysis time and sensitivity. Recently published analytical methods for the measurement of HP and its metabolites include the derivatization of HP [19—23]. Most of those assays avoid the use of heat and acid during the derivatization process, but, as a result, reaction times of up to 1 h and multiple-extraction procedures are required. These steps add more time to the analytical procedure and may compromise the specificity of the assay owing to the instability of HP. Methods employing both GC and HPLC have been described for the measurement of several HP metabolites [20, 21]. The assay presented in this communication offers the advantage of the simultaneous measurement of several proposed metabolites of HP by HPLC alone in a relatively simple and rapid manner.

The availability of this method for the analysis of hepatic HP metabolites should facilitate investigations dealing with the role of metabolism in the development of adverse reactions to HP. The microsomal metabolism of HP may be associated with the activation of the drug [24] and, in preliminary studies, we have used this HPLC assay to identify and quantitate the metabolites produced during the incubation of rat liver microsomes with HP. We have found that incubation of rat liver microsomes with HP in the presence of NADPH results in the production of four metabolites which co-chromatographed with P, PZ, TP and MTP (Fig. 7). The identities of these metabolites have been confirmed by mass spectroscopy. A major unknown metabolite with a retention time of 22 min was also produced. Very small amounts of metabolic products were present in the unincubated samples which result from the chemical degradation of HP prior to its removal from the incubation samples by extraction, but they do not compromise the specificity of the assay. The objectives of subsequent investigations will be to use the analytical method described in this communication to further study the metabolism of HP in vivo and in vitro in an attempt to determine the pathway(s) and product(s) responsible for the toxicity of HP.

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