In Vitro Kinetic Studies of the Reaction of Hydralazine and Its Acetone Hydrazone with Pyruvic Acid

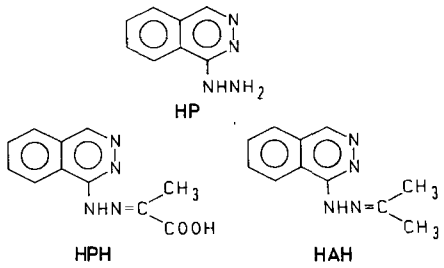
MASAHIRO IWAKI, TARO OGISO[x], AND YOSHIMASA ITO

Received December 29, 1986, from the *Faculty of Pharmaceutical Sciences, Kinki University, Kowakae 3-4-1, Higashi-Osaka, Osaka, Japan, 577.* Accepted for publication September 29, 1987.

Abstract □ To understand the reaction between hydralazine (HP) or its acetone hydrazone (HAH), a metabolite of HP and pyruvic acid, a new selective HPLC method for simultaneous determination of HP, HAH, and hydralazine pyruvic acid hydrazone (HPH) was developed. In vitro degradation of HAH and formation of HP and HPH were investigated at pH 7.4 and 37 °C in the presence or absence of pyruvic acid. Hydralazine degraded slowly according to an apparent first-order rate (7.46×10^{-2} h^{-1}). The degenerative reaction of HAH, accompanied by simultaneous hydrolysis to the parent drug HP, was also subject to apparent first-order loss (3.00×10^{-1} h^{-1}). In addition, HAH was partly converted to HP and HPH in the presence of pyruvic acid. For the formation pathway of HPH, a model that included the direct reaction of HAH with pyruvic acid and the secondary formation mediated by back-conversion to HP gave a better fit to the experimental data than the model consisting of the latter reaction only. About 10% of the HPH formed was generated by the direct reaction of HAH with pyruvic acid, based on the rate constants estimated. These results suggest that the formation of HPH is not all accomplished through back-conversion to HP.

Hydralazine (HP) has been used to treat hypertension by direct peripheral vasodilation. The metabolism of HP is complicated because HP can chemically react with endogenous compounds in addition to the enzymatic metabolism. Hydralazine readily forms various hydrazones with aldehydes and ketones. The pyruvic acid hydrazone (HPH) is identified as a major metabolite of HP in the systemic circulation,[1,2] and the acetone hydrazone (HAH) is qualitatively demonstrated to be a metabolite in human urine.[3] The hypotensive effect of these hydrazones is observed after their administration to rats, although the hydrazones are considerably less potent than parent drug, HP.[4,5] On the other hand, it has been reported that a relatively stable hydrazone, HPH, is inactive on hypertensive rabbits[6] and humans[7] and that HPH generated in plasma after administration of HAH may be due to back-conversion from HAH to HP.[8] In addition, Clementi et al.[9] have concluded that HP hydrazones are inactive, except when hydrolyzed to HP. However, it has not been clarified whether the hypotensive effect of HP hydrazones is attributed to their intrinsic activity or secondarily to back-conversion to the parent drug, HP. The lack of a selective assay for the separation and quantitation of HP and its hydrazones makes it difficult to exactly interpret the pharmacokinetic behavior and hypotensive effect of HP hydrazones. Because of the potential for conversion of some of the HP hydrazones back to HP, or a direct reaction of the hydrazones, like that of HP with *p*-anisaldehyde, during the assay process, the kinetic analysis of HP hydrazone (especially HAH) may be difficult (see the structural formulas of the compounds discussed). To clarify the pharmacokinetics of HP hydrazones, a selective assay for HP and HP hydrazones is, thus, absolutely required.

In this study, the in vitro formation kinetics of HPH from HAH and pyruvic acid and the degradation kinetics of HP and HAH at pH 7.4 were evaluated using a convenient, newly developed HPLC method for the simultaneous determination of intact HP, HPH, and HAH. The proposed assay procedure gave excellent selectivity for HP and its hydrazones, without derivatization.



Experimental Section

Materials—Hydralazine (HP) hydrochloride was kindly supplied by Japan Ciba-Geigy (Takarazuka, Japan). Phenacetin for an internal standard for the HPLC assay and sodium dodecyl sulfate (HPLC grade) for ion-pair chromatography were obtained from Nakarai Chemicals, Ltd. (Kyoto, Japan). Hydralazine pyruvic acid hydrazone (HPH) and HAH were synthesized according to the methods of Haegele et al.[10,11] The purity of the synthesized hydrazones was determined by GC-MS analysis of methanolic solutions containing 100 μg/mL of each hydrazone with or without a 1-μg/mL spike of HP. Comparison of the mass spectra revealed that the purity of hydrazones was >99.0% on a weight/weight basis. All the other chemicals were of reagent grade.

Analytical Procedure—A newly developed HPLC method was used for the simultaneous determination of HP, HPH, and HAH. An HPLC (model LC-6A, Shimadzu Corp., Kyoto, Japan) with a variable wavelength detector (Shimadzu model SPD-6AV) and a C_{18} reversed-phase column (Inertsil ODS, 5-μm particle size, 250 × 4.6 mm i.d., Gasukuro Kogyo, Inc., Tokyo, Japan) was used. The mobile phase consisted of acetonitrile:50 mM ammonium phosphate buffer (pH 4.0) containing 1.54 mM sodium dodecyl sulfate:tetrahydrofuran (35:65:1). The wavelength used was 254 nm. The operating temperature was 40 °C, and the flow rate was 1.0 mL/min. The peak areas were computed using an integrator (Shimadzu model C-R3A), and an internal standard was used for quantitation. To 100 μL of sample, 1 ng/μL of phenacetin in acetonitrile (100 μL) as an internal standard was added. The tube contents were then mixed on a vortex mixer. The mixture (80 μL) was immediately injected into the column. If a plasma sample was used, the supernatant was injected after centrifugation of the mixture at 12,000 rpm for 30 s. Figure 1 shows the chromatograms obtained by this method. Hydralazine pyruvic acid hydrazone (HPH), HP, phenacetin, and HAH gave retention times of 4.7, 6.9, 10.1, and 12.5 min, respectively. No degradation of HP hydrazones to HP was observed during the eluting process. The sensitivity, with routine detection limits of 0.1 μM, was obtained for each compound, and the responses of all three compounds versus the amount injected were linear in the 0.1–100-μM range. The intra-assay coefficients of variation for the assays of HP and its hydrazones ranged from 1 to 8% over the range of 0.5 to 50 μM, and the interassay variability ranged from 2 to 5%.

Degradation of Hydralazine and Its Acetone Hydrazone with Pyruvic Acid—The degradation of HP and HAH was measured in 12.5- or 25.0-μM solutions of HP or HAH, at pH 7.4 and 37 °C, in the presence of 0.1 mg/mL of gentamycin. The solutions of HP and HAH

0022-3549/88/0300-280$01.00/0

were freshly prepared in 0.1 M phosphate buffer (pH 7.4). After the addition of HP or HAH, the mixture was incubated at 37 °C, and samples were taken at selected intervals and immediately analyzed according to the HPLC method described above.

Reaction of Hydralazine and Its Acetone Hydrazone—The generation of HPH from HP or HAH and pyruvic acid was followed at 37 °C in the presence of 12.5 or 25.0 μM HP or HAH and 500 μM pyruvic acid buffered at pH 7.4. The solutions of HP and HAH were prepared as described above. By addition of the desired amount of HP or HAH to the buffer containing 500 μM pyruvic acid, the kinetic experiments were initiated. Sampling and analysis were identical to the method used in the experiment mentioned above.

Kinetic Modeling—In the present study, the major aim of modeling is to help in the interpretation of the estimates obtained. The proposed model of the reaction of HAH with pyruvic acid (Figure 2) consists of the following features. The first includes a combination of two parallel reactions for HPH formation, namely the direct formation of HPH from HAH and the hydrolysis of HAH to HP which forms HPH with pyruvic acid. The second is that both HP and HAH decompose to unidentified compound(s). On the other hand, further alteration of HPH was not considered, since the degradation of HPH under the conditions tested was neglibible.[4,12] It was postulated that the reactions of these compounds were adequately described by apparent or pseudo first-order kinetics. This led to a linear dynamic model, which, as a rule, was the simplest of all possible models. From the above considerations, the model presented in Figure 2 can be expressed mathematically with the following differential equations:

$$\frac{dC_{HAH}}{dt} = -(k_1 + k_2 + k_3)C_{HAH} \quad (1A)$$

$$\frac{dC_{HP}}{dt} = k_2C_{HAH} - (k_4 + k_5)C_{HP} \quad (1B)$$

$$\frac{dC_{HPH}}{dt} = k_3C_{HAH} + k_4C_{HP} \quad (1C)$$

In the above expressions, C_{HAH}, C_{HP}, and C_{HPH} denote the concentrations of HAH, HP, and HPH, respectively, with initial conditions of $C_{HAH} = C^0_{HAH}$, and $C_{HP} = C_{HPH} = 0$. Solving these differential equations, the concentration of each component in the mixture can be expressed as a function of time:

$$C^0_{HAH} = C_{HAH}\exp(-k_{d1}t) \quad (2A)$$

$$C_{HP} = \frac{k_2C^0_{HAH}}{k_{d1} - k_{d2}}[\exp(-k_{d2}t) - \exp(-k_{d1}t)] \quad (2B)$$

$$C_{HPH} = C^0_{HAH}\left[\frac{k_3k_{d2} + k_2k_5}{k_{d1}k_{d2}} - \frac{k_3k_{d2} + k_2k_5 - k_3k_{d1}}{k_{d1}(k_{d2} - k_{d1})}\exp(-k_{d1}t) - \frac{k_2k_5}{k_{d2}(k_{d1} - k_{d2})}\exp(-k_{d2}t)\right] \quad (2C)$$

where $k_{d1} = k_1 + k_2 + k_3$ and $k_{d2} = k_4 + k_5$.

The rate constants were estimated by nonlinear least-squares regression analysis (MULTI[13]) with a desk-top digital computer (PC-9801, NEC Corp., Tokyo, Japan). Data in all functions were weighted numerically equal.

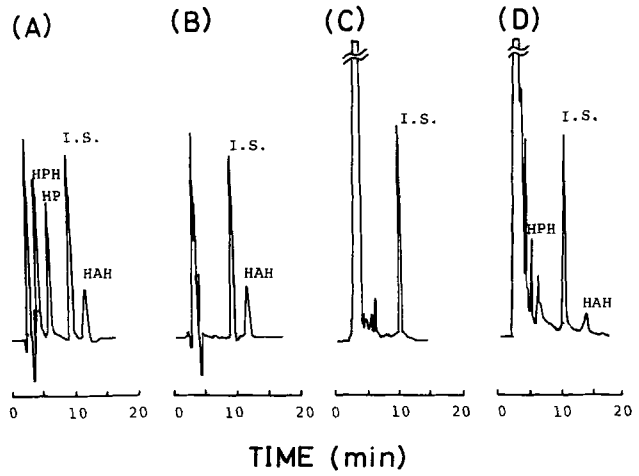


Figure 1—*The HPLC chromatogram of hydralazine (HP) and its pyruvic acid (HPH) and acetone hydrazones (HAH; see Experimental Section for assay conditions). Key:* (I.S.) *internal standard (phenacetin);* (A) *authentic compounds;* (B) *freshly prepared standard sample of HAH (25 μM);* (C) *plasma blank containing* I.S.; (D) *plasma obtained 40 min after an iv dose of HAH (10 mg/kg) to rat.*

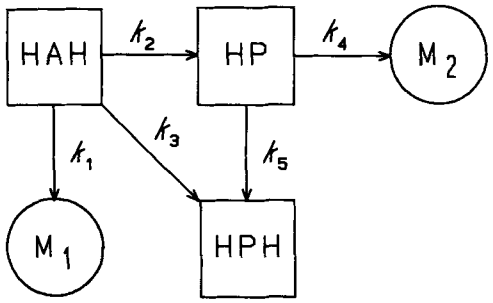


Figure 2—*The kinetic model proposed for conversion of hydralazine acetone hydrazone in the presence of pyruvic acid:* M_1 *and* M_2 *refer to the unidentical compound(s) derived from HAH and HP, respectively; the* k_i *values are apparent or pseudo first-order rate constants.*

Results and Discussion

Concentrations used in this study were high relative to those observed after usual HP dosing. Considering that the maximum plasma level of HP is 500 ng/mL,[14] the concentration ratio of pyruvic acid to HP in blood is ~22.[15] Therefore, the disappearance of HP and HAH and the formation of HPH were examined in a physiological concentration ratio of pyruvic acid to the drugs, although the rate constants obtained may not directly relate to the in vivo situation.

Degradation of Hydralazine and Its Acetone Hydrazone—Hydralazine is stable at acidic and neutral pH,[16] but in alkaline solution, it undergoes degradation to phthalazine.[17] To estimate the stability of HP at pH 7.4, a 12.5- or 25.0-μM solution of HP buffered at pH 7.4 was incubated at

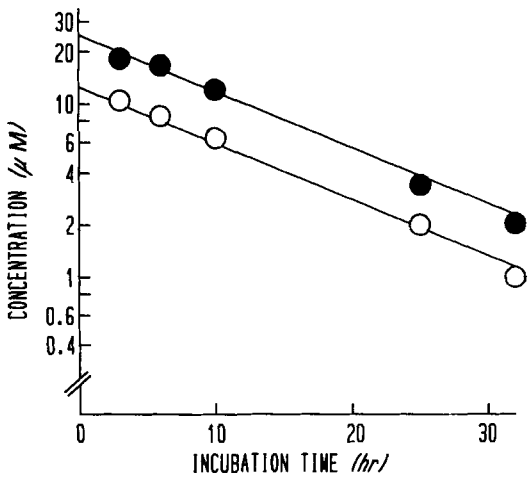


Figure 3—*Semilog plots for degradation of HP in 0.1 M phosphate buffer (pH 7.4) containing 12.5 μM (○) and 25.0 μM (●) HP as initial concentrations at 37 °C. Plots are the mean data (n = 3 preparations), and lines are the best fit values calculated using eq 3.*

37 °C. Figure 3 shows the semilog plots of the concentrations of remaining HP as a function of time. The plots show good linearity at both concentrations of HP, indicating that the disappearance of HP in aqueous solutions follows apparent first-order kinetics under the experimental conditions used. The degradation rate constant of HP was computed according to eq 3, and appeared to be independent of the concentration of HP. The calculated first-order rate constant was 7.46×10^{-2} ($\pm 5.03 \times 10^{-3}$) h^{-1}.

$$C_{HP} = C^0_{HP} \exp(-k_4 t) \quad (3)$$

Figure 4 depicts the concentration–time profile of the residual HAH and product HP. The semilog plots indicated that the degradation of HAH also followed apparent first-order kinetics. The degradation of HAH was followed by the generation of an appreciable amount of HP. The HAH hydrolysis to HP, as reported by Clementi et al.,[9] was clearly confirmed by our HPLC method. However, the sum amounts of HAH and HP in the medium were not equal to that of HAH added, indicating that the conversion of HAH to products other than HP was included. The time courses of disappearance of HAH and generation of HP can be expressed by:

$$C_{HAH} = C^0_{HAH} \exp[-(k_1 + k_2)t] \quad (4A)$$

$$C_{HP} = \frac{k_2 C^0_{HAH}}{k_1 + k_2 - k_4} [\exp(-k_4 t) - \exp(-(k_1 + k_2)t)] \quad (4B)$$

Equations 4A and 4B were used to simultaneously calculate k_1 and k_2 on the basis of the experimental data derived from the degradation of HAH. The degradation rate constant of HAH, the sum of k_1 and k_2, was 3.00×10^{-1} ($\pm 7.87 \times 10^{-3}$) h^{-1}. This value was four times higher than that of HP. The generation rate constant of HP from HAH, k_2, at pH 7.4 and 37 °C, was estimated as being 1.59×10^{-1} h^{-1}. The ratio k_2:($k_1 + k_2$) was 0.53, suggesting that about half of the HAH was hydrolyzed to HP under the conditions tested.

Formation of the Pyruvic Acid Hydrazone from Hydralazine—We have reported that HPH formation from HP and pyruvic acid follows second-order kinetics with a rate constant of 0.333 L/mol/s in the mixture of 30.86 μM HP and 710 μM pyruvic acid.[4] In this paper, the formation kinetics of HPH was simplified to pseudo first-order kinetics, since only one concentration of pyruvic acid, which was 20–40 times higher than that of HP, was used. To re-evaluate the HPH formation rate from HP and pyruvic acid under the previous conditions, HP was incubated at 37 °C and pH 7.4 in a solution containing 500 μM pyruvic acid. As shown in Figure 5, rapid appearance of HPH was observed as a result of the rapid and extensive reaction of HP with pyruvic acid. The formation rate constant was computed according to the following equation:

$$C_{HPH} = \frac{k_5 C^0_{HP}}{k_4 + k_5} [1 - \exp(-(k_4 + k_5)t)] \quad (5)$$

When fitting the data to the model, k_4 was fixed at 7.46×10^{-2} h^{-1}. The calculated formation rate constant of HPH from HP was 6.14×10^{-1} ($\pm 2.85 \times 10^{-2}$) h^{-1}, the largest of the other constants calculated. This result demonstrates that the reaction of HP with pyruvic acid occurs most easily, as reported by other workers.[12,17] The values for k_i, estimated separately, are summarized in Table I.

Possible Formation Pathway of the Pyruvic Acid Hydrazone from the Acetone Hydrazone—The acetone hydrazone was incubated at 37 °C and pH 7.4 in the presence of pyruvic acid. Figure 6 shows the time course of concentrations of

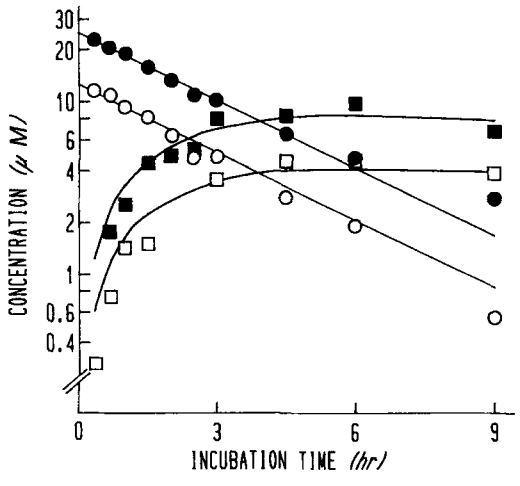


Figure 4—*Semilog plots for degradation of HAH (circles) and formation of HP (squares) in 0.1 M phosphate buffer (pH 7.4) containing 12.5 μM (open symbols) and 25.0 μM (closed symbols) HAH as initial concentrations at 37 °C. Plots are the mean data (n = 4 preparations), and lines are the best fit values calculated using eqs 4A and 4B.*

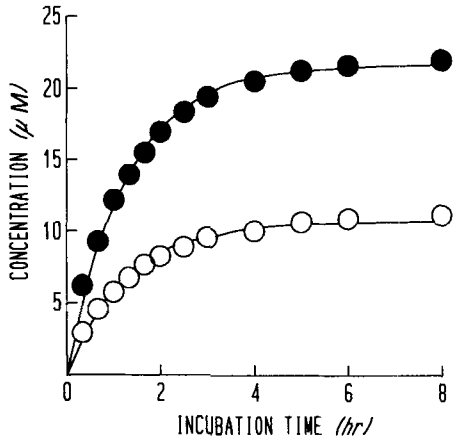


Figure 5—*Time course of HPH generated from 12.5 μM (○) and 25.0 μM (●) HP in 0.1 M phosphate buffer (pH 7.4) containing 500 μM pyruvic acid at 37 °C. Plots are the mean data (n = 4 preparations), and lines are the best fit values calculated using eq 5.*

Table I—First-Order Rate Constants[a] for the Alteration of Hydralazine (HP) and Its Acetone Hydrazone (HAH) at pH 7.4 and 37 °C in the Presence or Absence of Pyruvic Acid

Parameter[b]	Separate Fitting Estimate, h^{-1}[c]	Simultaneous Fitting Estimate, h^{-1}[d]
k_1	1.41×10^{-1}	1.10×10^{-1}
k_2	1.59×10^{-1}	1.62×10^{-1}
k_3	—	1.78×10^{-2}
k_{d1}	3.00×10^{-1}	2.90×10^{-1}
k_4	7.46×10^{-2}	6.48×10^{-3}
k_5	7.15×10^{-1}	6.63×10^{-1}
k_{d2}	7.90×10^{-1}	6.69×10^{-1}

[a] Calculated for solutions containing 12.5 or 25.0 μM HP and HAH and 500 μM pyruvic acid as initial concentrations (n = 3–5). [b] The k_i values are the rate constants shown in Figure 2; k_{d1} and k_{d2} are the disappearance rate constants of HAH and HP, respectively. [c] Obtained separately from each experiment. [d] Obtained simultaneously from the data for alteration of HAH in the presence of pyruvic acid, using the kinetic model shown in Figure 2.

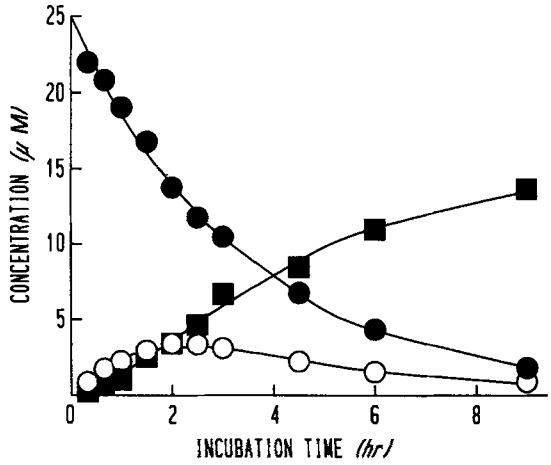


Figure 6—*Time course of degradation of HAH (●) and products, HP (○) and HPH (■), in 0.1 M phosphate buffer (pH 7.4) containing 25.0 μM HAH and 500 μM pyruvic acid at 37 °C. Plots are the mean data (n = 5 preparations), and lines are the best fit values calculated using eqs 2A–2C.*

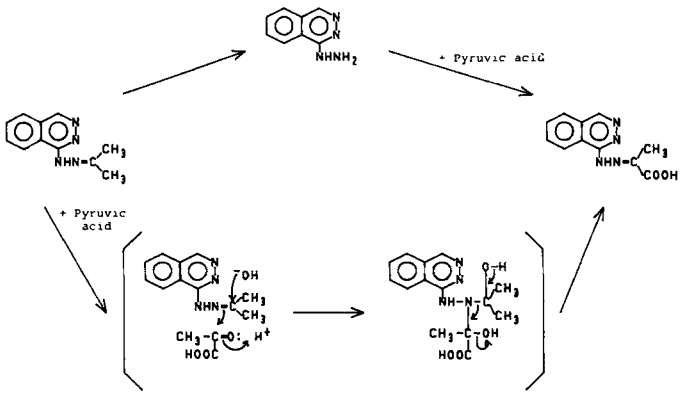


Figure 7—*Proposed conversion pattern of hydralazine acetone hydrazone (HAH) to hydralazine pyruvic acid hydrazone (HPH) in aqueous solution containing pyruvic acid.*

HAH and the products (HP and HPH) during the incubation of HAH with pyruvic acid. The acetone hydrazone (HAH) yielded HP and HPH on exposure to pyruvic acid. In the model presented in Figure 2, eqs 2A–2C were used to simultaneously calculate k_1–k_5, with the data derived from the degradation of HAH in the presence of pyruvic acid. The estimates for rate constants are shown in Table I. The rate constants from simultaneous fitting were close to the values obtained separately from each experiment. The value for k_4 obtained from simultaneous fitting was much smaller than that from separate fitting. This suggests that the decomposition of HP is negligible in this system. Based on the rate constants, ~90% of HPH formed was generated via HP formation.

The model presented in Figure 2 gave a better fitting compared with the sequential model (k_3 = 0 in the model in Figure 2) with back-conversion to HP, as judged from the lower Akaike's information criterion[18] (37.7 for the model in Figure 2 and 43.4 for the sequential model) and the residual sum of squares between the experimental and theoretical values (0.144 versus 0.387). Additionally, in the latter model, the k_4 converged value was negative (-4.61×10^{-2} h^{-1}), this being irrational. These results suggest a possibility of direct reaction of HAH with pyruvic acid. This is partially incompatible with the findings presented by Talseth et al.[8] and Clementi et al.[9] that HPH formation from HAH proceeds via HP formation. They discuss that oxidation of the methyl group of HAH to carboxylic acid is very unlikely. However, as shown in Figure 7, pyruvic acid (that is a more reactive electrophile than acetone) can exchange with the isopropyl group of HAH. Accordingly, the formation of HPH from HAH and pyruvic acid should be considered as a result of the combination of two parallel reactions, the direct transformation of HAH to HPH and the indirect formation mediated by back-conversion to HP, although contribution of the former to the overall formation of HPH was relatively slight. We have postulated, therefore, that HPH formation is not always by back-conversion from HAH to HP in vitro, and that the possibility of direct conversion in vivo may exist. The proposed HPLC method provided a rapid quantitative analysis for the conversive reaction of HP hydrazones.

References and Notes

1. Haegele, K. D.; Skrdlant, H. B.; Robie, N. W.; Lalka, D.; McNay, J. L. *J. Chromatogr.* **1976**, *126*, 517–534.
2. Wagner, J.; Faigle, J. W.; Imhof, P.; Liehr, G. *Arzneim.-Forsch.* **1977**, *27*, 2388–2395.
3. Haegele, K. D.; McLean, A. J.; duSouch, P.; Barron, K.; Laquer, L.; McNay, J. L.; Carrier, O. *Br. J. Clin. Pharmacol.* **1978**, *5*, 489–494.
4. Ogiso, T.; Iwaki, M.; Ohtsuki, N. *J. Pharmacol. Exp. Ther.* **1985**, *233*, 485–490.
5. Criscione, L.; Eichenberger, K.; Hedwall, P. R.; Schmid, K. *J. Cardiovasc. Pharmacol.* **1986**, *8*, 420–427.
6. Talseth, T.; Haegele, K. D.; McNay, J. L.; Skrdlant, H. B.; Clementi, W. A.; Shepherd, A. M. M. *J. Pharmacol. Exp. Ther.* **1979**, *211*, 509–513.
7. Reece, P. A.; Stafford, I.; Prager, R. H.; Wolker, G. L.; Zacest, R. *J. Pharm. Sci.* **1985**, *74*, 193–196.
8. Talseth, T.; McNay, J. L.; Haegele, K. D. *J. Cardiovasc. Pharmacol.* **1982**, *4*, 370–374.
9. Clementi, W. A.; McNay, J. L.; Talseth, T.; Haegele, K. D.; Ludden, T. M.; Musgrave, G. E. *J. Pharmacol. Exp. Ther.* **1982**, *222*, 159–165.
10. Haegele, K. D.; Skrdlant, H. B.; Talseth, T.; McNay, J. L.; Shepherd, A. M. M.; Clementi, W. A. *J. Chromatogr.* **1980**, *187*, 171–179.
11. Haegele, K. D.; Skrdlant, H. B.; Robie, N. W.; Lalka, D.; McNay, J. L. *J. Chromatogr.* **1976**, *126*, 517–534.
12. Reece, P. A.; Cozamanis, I.; Zacest, R. *J. Chromatogr.* **1980**, *181*, 427–440.
13. Yamaoka, K. *Analytical Method for Pharmacokinetics Using Computer*; Nankodo: Tokyo, Japan, 1984; pp 81–83.
14. Talseth, T. *Clin. Pharmacokinet.* **1977**, *2*, 317–329.
15. Documenta Geigy, *Scientific Tables*, 7th Ed.; Dien, K.; Lentrer, C., Eds.; J. R. Geigy, S. A. Basel, 1970; pp 607–608.
16. McIsaac, W. H.; Kanda, M. *J. Pharmacol. Exp. Ther.* **1964**, *143*, 7–13.
17. Reidenberg, M. M.; Drayer, D.; deMarco, A. L.; Bello, C. T. *Clin. Pharmacol. Ther.* **1973**, *14*, 970–977.
18. Yamaoka, K.; Nakagawa, T.; Uno, T. *J. Pharmacokineti. Biopharm.* **1972**, *6*, 165–175.