Binding of Pyridine Derivatives to Cytochrome P-450

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Abstract □ The spectral binding constants, K_s, of pyridine, methylpyridine, and dimethylpyridines were determined. The K_s values of the compounds were discussed in terms of mechanisms of possible bonding to cytochrome P-450.

Keyphrases □ Pyridine and derivatives—spectral binding to cytochrome P-450, constants determined □ Spectral binding—pyridine and derivatives to cytochrome P-450, constants determined □ Binding, spectral—pyridine and derivatives to cytochrome P-450, constants determined □ Cytochrome P-450—spectral binding to pyridine and derivatives, constants determined

Cytochrome P-450, a terminal oxidase responsible for the hydroxylation of many drugs and organic compounds (1, 2), exhibits two distinct types of spectral binding curves when the difference spectra are recorded in the presence of "substrates." Type I binding curves are thought to result from the interaction of a substrate with the enzyme at a site associated with hydroxylation (3), resulting in a perturbation of the enzyme that produces a difference spectrum with a peak in the 390-nm region and a trough in the 420-nm region (4). The binding of type II substrates involves the interaction of nonbonding electrons with the heme site (5–8), producing difference spectra with a peak in the 420-nm region and a trough at approximately 390 nm (4).

Many type II binding compounds have been identified including aliphatic alcohols, primary aliphatic and aromatic amines, pyridine, and metyrapone[1], the potent inhibitor of cytochrome P-450 (2). Water recently was proposed as the sixth ligand of the heme site with four ligands from the porphyrin ring and the other ligand from a sulfhydryl group (5). The nonbonding electrons of pyridine are thought to result in the displacement of water from the heme site, resulting in a pyridine-coordinated cytochrome P-450. This work describes the determination of the spectral binding constants for a series of methylpyridines.

EXPERIMENTAL

Adult male rats were injected with 80 mg/kg ip of phenobarbital sodium for 3 consecutive days, fasted for 24 hr, and then sacrificed by decapitation. The liver was removed, washed with 0.25 M sucrose, and then homogenized in 10 volumes of 0.25 M sucrose using a Potter–Elveihem glass tube and a polytef [2] pestle. The homogenized material was centrifuged for 15 min at 10,000×g, and the supernate was removed and centrifuged at 100,000×g for 1 hr to produce a microsomal pellet. The microsomal pellet was resuspended in 0.15 M KCl and recentrifuged at 100,000×g. This microsomal pellet was then resuspended in 0.05 M tromethamine buffer (pH 7.4), and the protein was adjusted to 1 mg/ml by the method of Lowry (9).

Difference spectra were recorded by placing 3 ml of the protein solution in both the sample and reference cells and then recording the baseline using a double-beam spectrophotometer[3] equipped with a scattered transmission accessory. After the baseline was established, 3-μl portions of substrate dissolved in tromethamine buffer were added to the sample cell and successive spectra were recorded. When the solubility of the substrate in tromethamine buffer was low, spectroanalyzed methanol was utilized to dissolve the compounds in the buffer.

The spectral binding constant, K_s, was determined by substituting the difference between the maximum and minimum absorbance in the spectrum for initial velocity in the classical enzyme substrate models (10).

The pyridine compounds were distilled prior to use, and their purity was checked by GLC.

[1] Metopirone, Ciba-Geigy.
[2] Teflon (du Pont).
[3] Beckman M6 Acta.

Table I—Comparison of Spectral Binding Constants (K_s) for Pyridine and Methylpyridines

Compound	K_s	Relative Activities	pKa of Conjugate Acid[a]
Pyridine	1.46×10^{-3}	1	5.3
2-Methylpyridine	6.15×10^{-3}	0.24	5.95
3-Methylpyridine	0.353×10^{-3}	4.14	5.85
4-Methylpyridine	0.166×10^{-3}	8.8	6.10
2,6-Dimethylpyridine	1.95×10^{-3}	0.75	6.72
2,4-Dimethylpyridine	0.945×10^{-3}	1.54	6.8
2,5-Dimethylpyridine	3.7×10^{-3}	0.39	6.55
3,4-Dimethylpyridine	0.089×10^{-3}	16.4	6.61
3,5-Dimethylpyridine	0.16×10^{-3}	9.13	6.34

[a] N. Ikekana, Y. Sato, and T. Maeda, *Pharm. Bull.*, **2**, 205 (1954).

RESULTS AND DISCUSSION

Three distinct factors should influence the binding of methylpyridines to the heme site: the basicity of the compound, the steric factors involved with the approach of the methylpyridines to the heme site, and the hydrophobic bonding of the methyl groups to the site as proposed by Jefcoate *et al.* (7). The instability of 2-methylpyridine and 2,6-dimethylpyridine complexes with formyl-substituted hemins (11) was attributed to steric inhibition of bonding, as was the instability of the boron trifluoride–2-methylpyridine complex (12). If the nonbonding electrons of pyridine are involved with coordination with the heme site of cytochrome P-450, introduction of a methyl group on the 2-position of pyridine should result in less binding of the compound to the heme site.

Table I shows the spectral binding constants for some methyl-substituted pyridines, the relative activities of the compounds as binders to cytochrome P-450, and the pKa values. The results support the concept of the nonbonding electrons of pyridine interacting with the heme site. In each case investigated, the activity of 2-methylpyridines was uniformly low. 2-Methylpyridine was approximately one-fourth as active as pyridine, and the activities of 2,4-dimethylpyridine and 2,5-dimethylpyridine were significantly lower than those of their parent compounds, 4-methylpyridine and 3-methylpyridine, respectively, indicating the importance of having an unsubstituted 2-position on the pyridine ring for maximal binding.

The discovery that 2,6-dimethylpyridine had a greater activity than 2-methylpyridine was unexpected and may be due to its basicity (pKa 6.72). The activity of 2,6-dimethylpyridine cannot be explained by a direct interaction between the nonbonding electrons and the heme site and may result from the transfer of electrons from the nitrogen to the heme site through a molecule of water.

A plot of log K_s for the 3- and 4-substituted pyridines against the pKa for the compounds shows a linear relationship between pKa and the binding activity ($r = 0.9817$). While this information cannot preclude the possibility of hydrophobic bonding, it strongly suggests that the basicity of the nitrogen is the dominant binding force in this series of compounds.

The consistently high activities of 4-methylpyridines suggests that appropriately substituted 4-pyridines may be potent binders of cytochrome P-450, a possibility now under investigation in these laboratories.

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Determination of Dextrorphan in Plasma and Evaluation of Bioavailability of Dextromethorphan Hydrobromide in Humans

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Abstract ☐ A method is described for the estimation of dextrorphan, a metabolite of dextromethorphan, in plasma. The bioavailability of dextromethorphan hydrobromide after 30 mg po, as measured by the concentration of total (free and conjugated) dextrorphan in the plasma, was determined in six human volunteers with this procedure.

Keyphrases ☐ Dextrorphan—fluorometric analysis, human plasma ☐ Dextromethorphan hydrobromide—bioavailability evaluated using fluorometric analysis of dextrorphan in human plasma ☐ Fluorometry—analysis, dextrorphan in human plasma ☐ Bioavailability—dextromethorphan hydrobromide evaluated using fluorometric analysis of dextrorphan in human plasma ☐ Antitussives—dextromethorphan hydrobromide, bioavailability evaluated using fluorometric analysis of dextrorphan in human plasma

Dextromethorphan hydrobromide, a widely accepted, nonnarcotic antitussive agent, is a common ingredient in many cough–cold preparations. Despite its clinical use for over two decades, a method for estimating its plasma levels in humans has not been published. Presently available analytical methods lack the sensitivity needed to determine its plasma levels after oral administration of the maximum permissible over-the-counter dose of 30 mg[1] (1).

The metabolism of dextromethorphan has been studied in several species (2–6). Following oral administration, this drug is rapidly metabolized; the principal metabolites are the *O*-demethylated product dextrorphan and its glucuronide and sulfate ester conjugates. In humans, dextrorphan [(+)-3-hydroxy-*N*-methylmorphinan], (+)-3-hydroxymorphinan, and traces of the unmetabolized drug were found in urine after oral administration of the drug (6). Enzymatic hydrolysis of urines from patients administered dextromethorphan yielded 40–50% of the drug and metabolites, mostly in the form of glucuronide and sulfate conjugates[1].

Because the blood levels of unmetabolized dextromethorphan were low, another approach was to measure the levels of the major metabolites. Dextrorphan was estimated in plasma and urine by paper chromatography (7), colorimetric determination of the methyl orange complex (8), and radiotracer techniques (9). These methods suffer from inherent disadvantages such as a lack of sensitivity and cumbersome operation. This report describes a sensitive and more convenient method for the determination of plasma levels of dextrorphan and its conjugates. Its applicability to bioavailability studies in humans also is demonstrated.

EXPERIMENTAL

Plasma Levels of Dextrorphan and Its Conjugates—Plasma (3.0 ml) was transferred into a glass-stoppered erlenmeyer flask (25 ml). The pH was adjusted to 5.5 with 3.0 *N* acetic acid, and an enzyme solution[2] (0.2 ml) containing 30,000 units of β-glucuronidase and 10,000 units of arylsulfatase was added. The flask was stoppered and incubated at 37° for 2 hr. The incubate was brought to room temperature, and the pH of the contents was adjusted to 9.5 by the addition of a saturated solution of sodium carbonate (0.6 ml).

Spectroscopically pure ethyl acetate (15 ml) was then pipetted into the flask, and the contents were shaken for 20 min on a mechanical shaker. The mixture was transferred quantitatively into a 30-ml centrifuge tube, and the extract was separated by centrifuging at 3000 rpm for 15 min. Then an aliquot (12 ml) of the supernate was transferred into a stoppered 50-ml erlenmeyer flask containing 3.0 ml of 1.0 *N* HCl, and

[1] B. A. Koechlin and F. Rubio, work cited in Ref. 1.
[2] Glusulase, Endo Laboratories, Garden City, NY 11530.