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EFFECT OF SALT CONCENTRATION ON THE ACTIVITY OF LIVER MICROSOMAL AND cDNA-EXPRESSED HUMAN CYTOCHROMES P450

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Abstract

The effect of ionic strength on the activity of cDNA-expressed and human liver microsomal cytochromes P450 was studied. Ionic strength was varied by manipulating the amount of potassium phosphate buffer in the incubation. These data show that for a specific enzyme, the ionic strength optimum did not depend on the source of enzyme - a specific liver microsomal or cDNA-expressed enzyme (produced in human lymphoblasts or insect cells with and without cytochrome b_5) showed similar salt dependencies. However, the optimum salt concentration differed among enzymes. CYP2C9 and CYP2C19 were more active at low salt concentrations (25 to 50 mM) while CYP1A2 and CYP3A4 were more active at high salt concentrations (100 to 150 mM). These observations imply that choice of salt concentrations for *in vitro* incubations can affect not only the overall rates of metabolism, but also the proportion of total metabolism catalyzed by different enzymes.

Introduction

Cytochromes P450 (CYP) are the principal enzymes for the oxidation of drug, environmental pollutants and other xenobiotics. This enzyme system consists of many distinct forms of the enzyme. All mammalian forms are membrane bound and found in many tissues, but at highest levels in liver. A principal function of the cytochrome P450 system is to convert lipophilic molecules into more water soluble forms which are more readily excreted from the body.

In vitro analysis of cytochrome P450-mediated metabolism is now routinely used in drug discovery and preclinical drug development. However, the specific incubation conditions (e.g. buffer type and concentration) vary widely among laboratories. The effect of buffer concentration on drug-metabolizing cytochrome P450 activities has not been extensively investigated. In the present study, we examine the effect of potassium phosphate buffer concentration, on rates of substrate metabolism by CYP1A2, CYP2C9, CYP2C19, CYP2D6 and CYP3A4.

Materials and Methods

Microsomes. Microsomes were obtained from Gentest Corp. (Woburn, MA) from metabolically competent human B-lymphoblastoid cell lines that stably express human cytochromes P450, from baculovirus-infected insect cells (Supersomes') or pooled human liver microsomes.

Cytochrome P450 Assays. Assays were performed in a final volume of 0.2 ml containing 1.3 mM $NADP^+$, 3.3 mM glucose-6-phosphate, 0.4 units/ml glucose-6-phosphate dehydrogenase, 3.3 mM $MgCl_2$, and either 10, 25, 50, 100, 150 or 200 mM potassium phosphate buffer, pH 7.4. Substrate and literature references for the assays were as follows: CYP1A2, 200 µM phenacetin (Butler et al., 1989); CYP3A4, 200 µM testosterone (Waxman et al., 1983); CYP2C9, 40 µM (Leeman et al., 1993); CYP2C19, 100 µM [^{14}C]-(S)-mephenytoin (sp. act. = 6 mCi/mmole, Wrighton et al., 1993); CYP2D6, 25 µM ($\pm$)-bufuralol (Kronbach et al., 1987). Enzyme concentrations and incubation times were selected to give less than 10% conversion into the measured metabolite to ensure first order reaction rate kinetics. Incubations were carried out for at 37°C.

The reactions were stopped by the addition of acetonitrile (CYP1A2, CYP2C19 and CYP3A4), 94% acetonitrile - 6% glacial acetic acid (CYP2C9), or perchloric acid (CYP2D6). After cooling on ice for 5 min. the incubations were centrifuged at 10000 x g for 3 minutes and aliquots removed for further analysis.

HPLC Analysis. The HPLC system consisted of Waters Model 510 pumps, a Waters Model 717 plus Autosampler, and a Waters Model 486 tunable absorbance detector or a Model 996 photo diode array detector. This system was controlled by an IBM-compatible computer using Waters Millennium Version 2.15 software. Instead of absorbance detectors, a Packard Radiomatic Flo-One flow scintillation analyzer was used to detect peaks in the 2C19 assay, and a Waters 474 fluorescence detector was employed (excitation - 252 nm; emission - 302 nm) in the 2D6 assay. Aliquots of supernatant from all were injected onto a Supelco 4.6 mm x 250 mm Nucleosil C18 5 µm HPLC column (Bellefonte, PA) and developed at 45°C with a flow rate of 1 ml/min. The HPLC column was protected by a Waters 3.9 x 20 mm 60A 4 µm Sentry guard column. Peaks from the CYP2C9 and CYP2D6 assays were developed with a methanol/acetonitrile-H_2O-perchloric acid mobile phase; the other HPLC analyses employed an aqueous methanol/methanol mobile phase.

Data Analysis. The results were obtained from at least two separate experiments for each salt concentration. Data are expressed as % activity compared to salt concentration which gave the highest activity.

Results

The effect of potassium phosphate concentration on the catalytic activities of five human cytochrome P450s are shown in Figures 1 to 5. Three or four enzyme sources were used per cytochrome P450 form analyzed: cDNA-expressed material derived from stably transfected human lymphoblasts ("Lymphoblast"), pooled human liver microsomes ("Liver"), cDNA-expressed material derived baculovirus infected insect cells without added cytochrome b_5 ("Insect") and with cDNA-expressed cytochrome b_5 ("Insect+ cytochrome b_5").

Figure 1. CYP1A2 and Phenacetin O-Deethylase

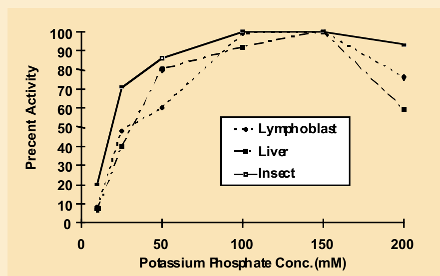


Figure 2. CYP2C9 and Diclofenac 4'-Hydroxylase

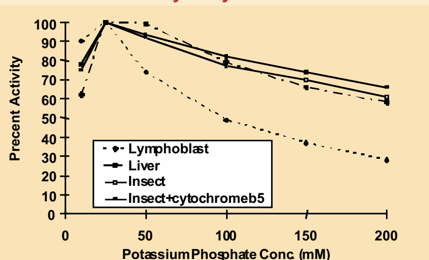


Figure 3. CYP2C19 and (S)-Mephenytoin 4'-Hydroxylase

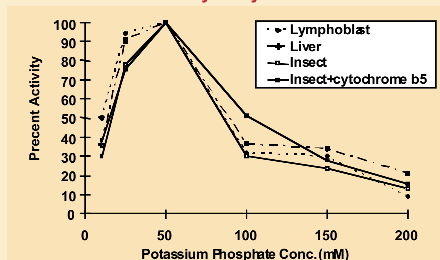


Figure 4. CYP2D6 and Bufuralol 1'-Hydroxylase

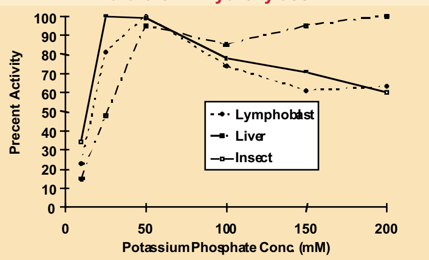
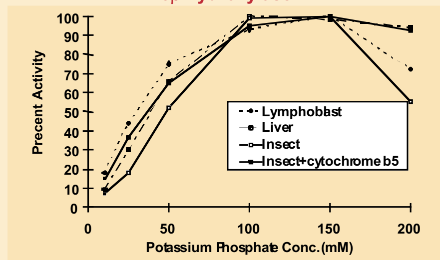


Figure 5. CYP3A4 and Testosterone 6β-hydroxylase



Conclusions

1. The five, major, human drug metabolizing cytochromes P450 differ in the potassium phosphate concentration necessary for maximal activity.
2. CYP2C9 and CYP2C19 are most active at low potassium phosphate concentrations while CYP1A2 and CYP3A4 prefer higher potassium phosphate concentrations. CYP2D6 is active over a broad range of potassium phosphate concentrations.
3. In general, the optimal (or near optimal) potassium phosphate concentrations for a particular enzyme is relatively broad. The exception is CYP2C19 where the optimum is relatively narrow.
4. For a specific enzyme, the potassium phosphate dependence is qualitatively similar regardless of enzyme source (cDNA-expressed or human liver).
5. The use 50 mM potassium phosphate should provide adequate activities for drug-metabolizing cytochromes P450.

References

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