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Determination of Kow Values for a Series of Aryl Glucuronides

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Polynuclear aromatic hydrocarbons (PAHs) are a class of hazardous contaminants in the aquatic environment that readily accumulate in animals. We have recently become interested in understanding the formation, distribution, and elimination of phase II metabolites of PAHs in fish (McKim et al. 1993) in support of the U.S. EPA's hazardous chemical risk assessment programs. Glucuronides are one of the important phase II metabolites and are formed by the conjugation of glucuronic acid with phase I metabolites of PAHs, hydroxylated PAHs (Clarke et al. 1991). The commercial availability of aryl glucuronides for study is, however, limited. We have, therefore, prepared a series of para substituted phenyl glucuronides, and wish to report a simple yet effective sample cleanup procedure for their isolation from microsomal incubation solutions, and features of ultra violet (UV) and electrospray ionization mass spectrometric (ESI/MS) data for their structural characterization.

An important parameter in toxicokinetic modeling is the octanol/water partition coefficient (Kow). This parameter has often been used to predict the accumulation of contaminants from water to fish (Klamer and Beekman 1995); however, few Kow values are available for modeling the behavior of phase II metabolites within an animal. Therefore, Kow values for the synthesized glucuronides, along with a few commercially available glucuronides, were determined using reversed-phase high performance liquid chromatography (RP-HPLC). The measured values were compared to those predicted by a substituent additive model, CLOGP (Leo and Weinninger 1988). An assessment of this data is presented.

METHODS AND MATERIALS

Phenol, uracil, p-aminophenol, hydroquinone, p-cresol, β-naphthol, p-fluorophenol, p-chlorophenol, p-methoxyphenol, p-t-butylphenol, and p-phenylphenol, were obtained from Chem Services (West Chester, PA). Phenyl glucuronide , p-nitrophenyl glucuronide, β-naphthyl glucuronide, 6-bromo-2-naphthyl glucuronide, trichloroacetic acid (TCA), formic acid, glycine, ammonium acetate, uridine 5-diphosphoglucuronic acid (UDPGA) and BSA were obtained from Sigma-Aldrich Chemical Co. (St. Louis, MO). Organic solvents were obtained from Fisher Scientific (Fair Lawn, NJ). All reagents were of the highest purity available.

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Rainbow trout (*Onchorynchus mykiss*) liver microsomes were prepared and characterized as previously described (Dady et al. 1991). Aryl glucuronides were prepared and purified in the following manner. Trout liver microsomes (500 µL, 8-10 mg protein), $MgCl_2$ (100µL of 100mM), UDPGA (100µL of 100mM), and a substituted phenol (100µL of 100mM in Tris-HCl (100mM, pH 7.6)) were combined, diluted to 1.5 mL with Tris-HCl (100mM, pH 7.6) and incubated with gentle mixing for 24 hr at 25° C. Protein was removed from the incubation reactions by the addition of either TCA (200µL, 0.6M containing glycine (0.3M)) or ice-cold ethanol (1 vol) followed by centrifugation (10min, 10,00Xg). The supernatant was transferred to a prewashed Quaternary Amine anion exchange (3mL) and/or a C_{18} Reversed Phase column (3mL) (J.T. Baker, Phillipsburg, NJ), and eluted with 0.1% formic acid in water (1-3mL) followed by 0.1% formic acid in methanol/water (5-50%, v:v, 1-6mL). One mL fractions were collected.

Glucuronide characterization was done using a Nouveau Gold HPLC system fitted with anUltrasphere C18 5µ RP-HPLC column (2X250mm) and a photodiode array UV detector (Beckman Instruments, Fullerton, CA). The eluant was either A:1% methanol, B:91% methanol or A:1% acetonitrile, B:51% acetonitrile, which were buffered at 0.5, 1.0, 2.5, 5.0, or 20.0 mM sodium phosphate (pH 6.8-7.0,1:1, v:v, monobasic:dibasic). The elution gradient was 100%A to 100%B in 15, 30, 60, 90, or 120 min. Solvent flow was 120µL/min, at 22-23°C. Glucuronide standards were 1mM. The injection volume was 20µL. For mass spectrometric analyses, the HPLC was connected to a Finnigan-MAT (San Jose, CA) TSQ-700 triple quadrupole mass spectrometer fitted with an ESI source (3.5 KV). The mobile phase contained 0.1% formic acid and 1.0mM ammonium acetate. MS/MS product ion spectra were generated using 1torr Argon, with a collision energy of 25V.

Calibration of the HPLC column for Kow determinations was done by regressing the Log of the shake flask Kow value (Hansch and Leo 1979) with the Log of the measured HPLC capacity factor for each of six calibration standards, uracil, p-aminophenol, hydroquinone, phenol, p-cresol, and β-naphthol. Phenyl phosphate was used to establish column void volume. Phenyl phosphate and p-cresol were added to each glucuronide solution as HPLC elution markers. Calculated Kow values were obtained using CLOGP (Leo and Weinninger 1988).

RESULTS AND DISCUSSION

Methods for the synthesis and characterization of the para-substituted phenyl glucuronides were developed using phenol to produce phenyl glucuronide. After protein precipitation, a typical average yield was 28.5% (n=5). The SPE column cleanup procedures resulted in very little product loss. The anion exchange column was found to be very effective for the separation of glucuronides with low Kow values from UDPGA, and the C_{18} reversed-phase SPE column effectively separated glucuronides with higher Kow values from their starting phenol. An example chromatogram, ES/MS spectrum, and UV spectrum for p-chlorophenyl glucuronide

are shown in Figure 1. The photodiode array UV spectra for each glucuronide showed two adsorption peaks, 220±5 and 270±5nm. The adsorption at 220nm was generally stronger; however, 270nm often has less interferences and thus may be more useful for detecting phenolic glucuronides. When ammonium acetate (1.0 mM) was added to the HPLC mobile phase for the ESI/MS analyses, each compound gave an intense ion at $(M+18)^+$ (ammonium ion adduct). Liberato et al. (1983) reported that the thermospray ionization spectrum of glucuronides showed an ion for the ammoniated glucuronic acid fragment at m/z 194. This ion was also observed in the ESI/MS/MS product ion spectra of phenyl-, p-nitrophenyl-, and naphthyl glucuronides (data not shown). Precursor ion scanning using m/z 194 may then be an very useful technique for the characterization of unknown glucuronides.

An excellent review of the theory of RP-HPLC for the determination of Kow values was presented by Braumann (1986). He describes the role of both the mobile and stationary phases in the retention of analytes, and presents correlations between HPLC capacity factors and Kow. Further he presents a discussion of the concept of Kw, the Kow value obtained by extrapolation of Kow values obtained using isocratic chromatography at various high percentages of organic modifier in the mobile phases to an expected value at 100% water. This concept, along with additional discussions of the influence of pH and ion-paring on Kow have more recently been reviewed (Hsieh and Dorsey 1993, Lambert 1993 and Valko et al. 1993). In 1997, Finizio et al. (1997) presented a review of the application of RP-HPLC to Kow determinations with respect to OECD guidelines. OECD recommends (Klein et al. 1988), among other things, 1. use of calibration standards with well established Kow values, 2. calibration with at least six standards, and 3.elution with at least 25% water. All of these reviews and recommended guidelines have generally been for assessing compounds of a wide variety of chemical classes with high Kow values. In this study, however, we focus on only one class of compounds, highly water soluble phase II metabolites. Analytical procedures, therefore, were less well established and required some development.

The criteria for selection of column calibration compounds was that they should be similar to the glucuronides, i.e. highly water soluble, at least partially aromatic, and weakly acidic. The series p-aminophenol, hydroquinone, phenol, p-cresol, and β−naphthol, which have Log Kow values of 0.04, 0.55, 1.48, 1.96. and 2.71 (averaged values, Hansch and Leo 1979) respectively, was selected. Uracil was also selected because it has a low Log Kow value, - 1.05. The HPLC mobile phases were buffered at neutral pH (1mM sodium phosphate, pH 6.8-7.0) to simulate the pH of living cells or blood. The OECD recommends that the analyses be done at a pH two log units below the pKa of the analyte (Klein et al. 1988). CLOGP estimates the pKa for all of the glucuronides to be about 3.0. Chromatography two log units lower (pH 1.0) is undesirable because, 1. it is not the pH of living organisms, 2. very few HPLC columns can survive extended exposure to such low pH values, and 3. glucuronides in solutions with a pH much greater than or less than neutral may hydrolyze. Methanol was chosen as the mobile phase organic modifier because it has been most

frequently used by other researchers reporting Kow values, and it is the most "water like" organic solvent (Lambert 1993). A linear gradient rather than an isocratic elution was chosen because analysis times could be shortened, and gradient analyses have been shown to predict Kow with a precision of better than 0.5 Log units (Klamer and Beekman 1995). It was important, however, to establish appropriate gradient conditions. To do this, the percent methanol in the mobile phase was changed (1-91%) at various rates from 0.75 to 6.0%/min, and the Kow value for two test glucuronides (phenyl- and naphthyl-) were plotted *vs* the percent methanol in the mobile phase at the time of analyte elution (Figure 2.). The Kow value for phenyl glucuronide was found to be insensitive to the change in percent methanol, eluting with 49.5% methanol in a 6%/min gradient and 6.7% methanol in a 0.75%/min gradient, but having a Log Kow value of -0.69 in each case. Naphthyl glucuronide, however, was found to be sensitive to the change in percent methanol, eluting in 76.2% methanol in a 6%/min gradient with a Log Kow value of 1.10 which increased to 1.39 when it eluted in 21.9% methanol from a 0.75%/min gradient. Because Log Kow values for both compounds changed little when the gradient was slowed from 1.0 to 0.75%/min, and because laboratory time could be saved using the faster gradient, the 1.0%/min gradient was selected to be used for the determination of Kow for the other glucuronides. Values reported are as measured, rather than extrapolated to Kw. Repeated analysis of naphthyl glucuronide showed a variability of ±0.01 log units, with a percent relative standard deviation of 0.16% (n=7). Results for the remaining glucuronides are presented in Table 1 (mean values, n=3). The measured values reported for p-aminophenyl- and p-hydroxyphenyl glucuronides are presented as estimated less than values. This is because they eluted between the column void volume marker, phenyl phosphate, and the first eluting standard, uracil. We were not able to obtain a Kow value for phenyl phosphate from the literature, nor was CLOGP able to calculate a value for this compound. Having a Kow value for phenyl phosphate may have allowed us to better estimate the Kow value of these two glucuronides. Log Kow values for the other glucuronides ranged from -0.69 for phenyl glucuronide to +1.79 for p-phenylphenyl glucuronide.

Glucuronide Kow values were evaluated in two ways, by comparison to their Kow values calculated by CLOGP, and by correlation to Kow values of a series of analogously substituted aromatic hydrocarbons (Table 1.). Measured and calculated Kow values were highly correlated (r^2=0.9402); however, calculated values were consistently lower, averaging 0.86 units lower. Similarly, a good correlation was found between the glucuronide Kow values and those for the aromatic hydrocarbons (r^2=0.9288).

Phenyl- and naphthyl glucuronide were also chosen for an assessment of ion-pair formation upon Kow. In an organic solvent-water system, an organic acid may partition into the organic phase as either a free anion, or as an neutral ion-pair, which will affect the apparent Kow of the acid (Strathmann and Jafvert 1998). The Kow values for the two test compounds were determined again using a methanol gradient of 1%/min, however, the buffer (sodium phosphate) concentration was first lowered

Glucuronide	Log Kow Values		
phenyl	HPLC measured*	CLOGP calculated**	Aromatic Hydrocarbon***
amino	<-2.0	-1.97	0.90
hydroxy	<-2.0	-1.67	1.48
hydro	-0.69	-1.18	2.10
nitro	-0.45	-1.14	1.84
fluoro	-0.42	-0.90	2.27
methoxy	-0.01	-1.09	2.08
methyl	0.37	-0.53	2.58
chloro	0.45	-0.33	2.49
t-butyl	1.73	0.80	4.11
phenyl	1.79	0.80	4.06
naphthyl			
hydro	1.39	-0.01	3.33
6-bromo	1.97	0.93	4.18

* HPLC gradient 100%A to 100%B at 1.0%/min, A=1% methanol, B=91% methanol, pH 6.8-7.0, 1.0mM sodium phosphate.** Leo and Weinninger 1988.*** Hansch and Leo 1979.

Table 1. HPLC determined and CLOGP calculated Log Kow values for a series of aryl glucuronides, along with Log Kow values for analogous substituted PAHs.

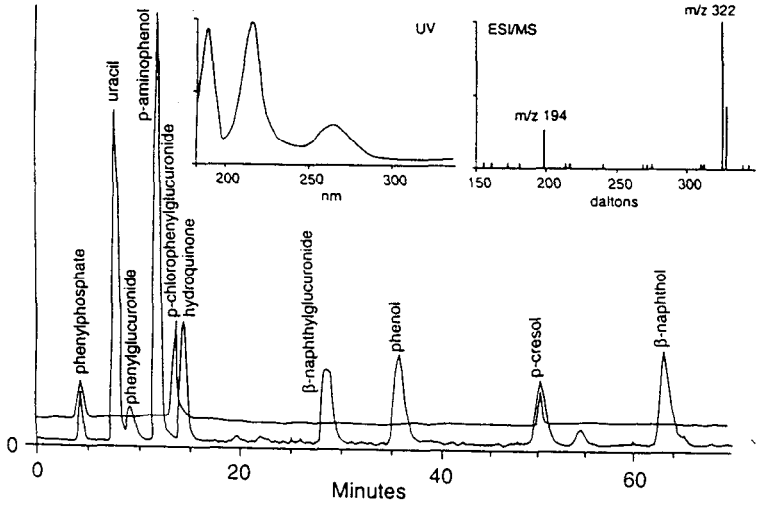

Figure 1. Chromatogram of HPLC column calibration compounds with phenyl- and naphthyl glucuronide (lower trace) and p-chlorophenyl glucuronide (upper trace). Chromatographic conditions were 1-91% methanol at 1 %/mm, 1 mM sodium phosphate, pH 6.8. UV spectrum (left insert) and ESI/MS (right insert).

to 0.5mM and then raised to 25mM for each determination. The three Kow values for phenyl glucuronide were - 1.20, -0.69, and -0.11, while for naphthyl glucuronide the values were 1.12, 1.40, and 1.61, for 0.5, 1.0 and 2.5mM, respectively (Figure 3.). This experiment showed that Kow for the anionic glucuronides was very sensitive to ion-pair formation with cationic sodium. The concentration of sodium in blood of rainbow trout has been determined to be about 145mM (McDonald and Milligan 1992). Kow values for either phenyl or naphthyl glucuronide can not be determined at such a high concentration of sodium because sodium phosphate will begin to come out of solution at concentrations above about 5mM in mobile phase B (91% methanol). By using a stronger organic modifier, such as acetonitrile, it is possible to decrease the percent of organic modifier in mobile phase B while keeping the retention time for analytes approximately the same. The concentration of the buffer salt in mobile phase B may then be increased, thus allowing the determination of Kow values to be done at higher concentrations of sodium. It was necessary, however, to first assess the effect of acetonitrile on Kow. Using mobile phases A:1% acetonitrile, and B:51% acetonitrile, containing 1mM sodium phosphate, Kow values for phenyl and naphthyl glucuronide were assessed at different percentages of acetonitrile in the mobile phase at the time of elution of the analyte in an experiment similar to that conducted with methanol (Figure 2.). Log Kow values for phenyl glucuronide eluting between 4.3 and 26.2% acetonitrile increased just slightly from -0.66 to 0.78, and were similar to the values obtained using methanol. Log Kow values for naphthyl glucuronide were lower than those obtained using methanol, ranging from 0.68 at 40.8% acetonitrile to 1.16 at 13.0% acetonitrile. As with the methanol system, Kow values did not change much when the gradient was slowed from 1.0 to 0.75%/min. To be comparable to the methanol data, a 1%/min gradient was then chosen to continue the ion-pair formation study. The concentrations of sodium used were 0.5, 1.0, 2.5, 5.0, and 20.0mM. Results are shown in Figure 3. For both of the test glucuronides the change of Log Kow between 0.5 and 5.0mM was large, going from -1.08 to -0.04 for phenyl glucuronide, and from 0.91 to 1.49 for naphthyl glucuronide, but then became asymptotic at 0.35 and 1.61, respectively, by 20mM.

In summary, Kow values presented in Table 1 were generated using instrumental conditions that should be easily reproducible in other laboratories so that partitioning data for other glucuronides may be compared to those presented here. The measured values seem reasonable because they correlate well with mathematically calculated values; are approximately 2.0-2.5 log units lower than the values for precursor aromatic hydrocarbons; and, correlate with Kow values for structurally similar aromatic hydrocarbons. Kow values for the glucuronides were found to be very sensitive to ion-pair formation, which may become very important if partitioning needs to be determined in a matrix such as blood where variations the concentration of cationic species such as Na^{+} and Ca^{++} may become important in the complexation with anionic organic species.

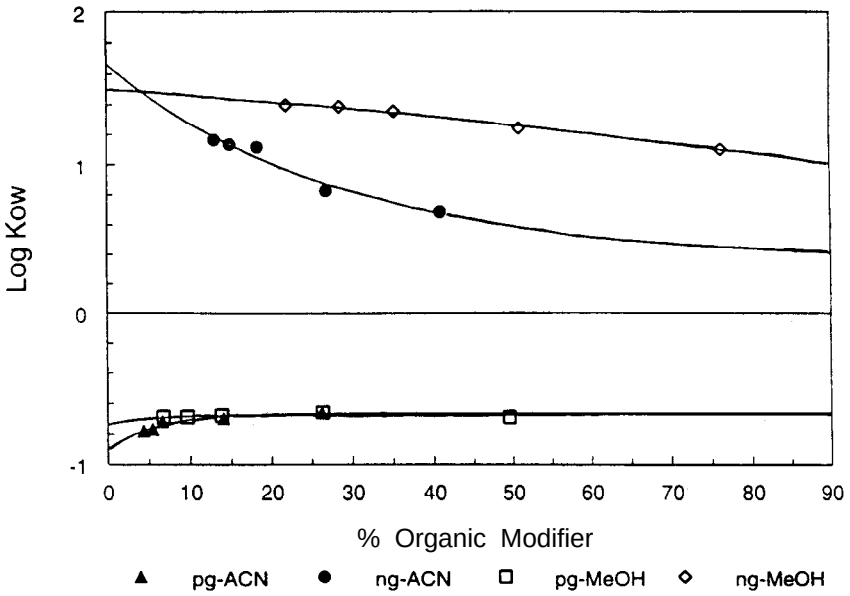

Figure 2. Log Kow values for phenyl- (pg) and naphthyl glucuronide (ng) *vs* percent of organic modifier in the HPLC mobile phase at the time of elution of the glucuronide. Data generated from a linear gradient (100%A: 1% methanol (MeOH) or 1% acetonitrile (ACN) to 100%B: 91% MeOH or 51% ACN, respectively, in 15, 30. 60, 90, or 120 min.

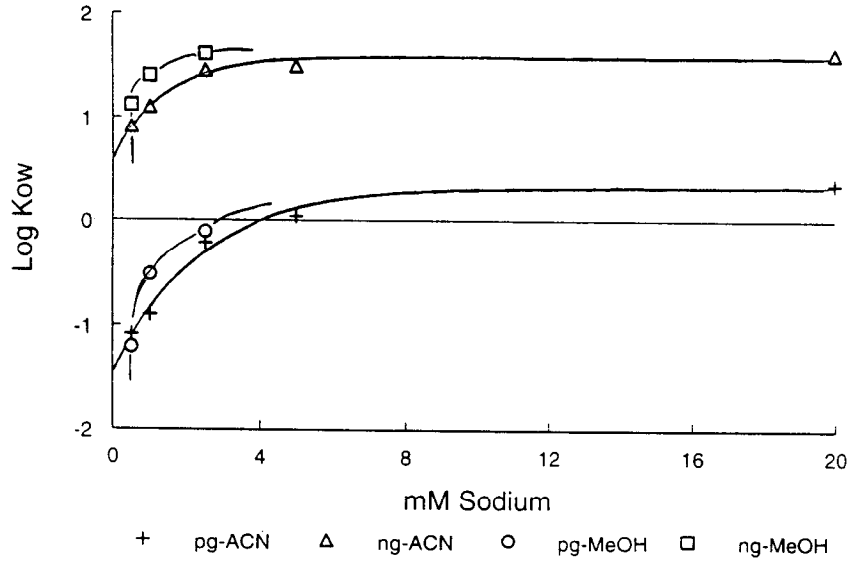

Figure 3. Log Kow values for phenyl- (pg) and naphthyl glucuronide (ng) eluted with either methanol or acetonitrile *vs* sodium phosphate concentration in the HPLC mobile phase. The percent organic modifier change in the mobile phase was 1 %/min Methanol changed from 1-91%. and acetonitrile changed from 1-51%.

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