Determination of the *N*-Hydroxy and Phenolic Metabolites of Drugs Derived from Acetanilide by *O*-Alkylation and Selected Ion Monitoring

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Metabolism of para alkoxy-substituted acetanilide by liver microsomal cytochrome P450 in vivo and in vitro can result in *O*-dealkylation and *N*-hydroxylation of the parent compounds. To study in vitro metabolism of para substituted analogues of acetanilide, a method was developed to simultaneously quantitate the *O*-dealkylated and *N*-hydroxylated para alkoxy-substituted acetanilides. This method uses *O*-alkyl derivatization of the metabolites in combination with gas chromatography/mass spectrometry and the technique of selected ion monitoring. The procedure should be applicable for analysis of phenolic and *N*-hydroxy metabolites of various drugs.

p-Methoxyacetanilide (methacetin) is the methyl analogue of *p*-ethoxyacetanilide (phenacetin), which is used as an analgesic and antipyretic drug. Metabolism of para substituted acetanilides by liver microsomal cytochrome P450 in vivo and in vitro results in *O*-dealkylation and *N*-hydroxylation of the parent compounds (Figure 1) (*1–3*). Although the *N*-hydroxy metabolites quantitatively represent a small percentage of the total metabolism, these types of compounds have been implicated as proximate mediators in several types of tissue toxicity; e.g., hepatic necrosis, nephropathy, methemoglobinemia, and various blood dyscrasias (*4, 5*). To study in vitro metabolism of *p*-substituted analogues of acetanilide and the mechanism by which the metabolites are formed, we required a method to simultaneously quantitate the enzymatic products of *p*-methoxyacetanilide, *p*-hydroxyacetanilide, and *N*-hydroxy-*p*-alkoxyacetanilide (Figure 1). Several good techniques are available for quantitating *p*-hydroxyacetanilide including colorimetry (*6*), gas chromatography (GC) (*7*), high pressure liquid chromatography (HPLC) (*8*), and gas chromatography/mass spectrometry, (GC/MS) in conjunction with the technique of selected ion monitoring (SIM) (*9*). However, quantitation of *N*-hydroxylated compounds such as *N*-hydroxy-*p*-methoxyacetanilide has been difficult because of their chemical instability and the low levels produced. Methods that have been used are colorimetry (*10*), paper chromatography with UV detection (*11*), thin-layer chromatography (TLC) in combination with radioisotopically labeled compounds (*12, 13*), GC using trimethylsilyl derivatives (*14, 15*), and HPLC (*16, 17*). All of these assays have certain limitations. For example, the colorimetric and spectrophotometric procedures lack specificity (e.g., N=O is not distinguished from N–OH) and are not very sensitive with a lower limit of detectability being 1 μg (*10*). We found that the trimethylsilyl derivatives of the *N*-hydroxylated derivatives used for GC analysis are unstable and give unreliable results. We describe here a specific method for analysis of *N*-hydroxylated para alkoxy-substituted and *p*-hydroxylated acetanilides which uses GC stable derivatives. Moreover, the use of various alkyl iodides allows a variety of *O*-alkyl derivatives to be formed so that interference from superimposition with peaks arising from the background reaction mixture can be systematically avoided.

EXPERIMENTAL

Reagents. *p*-Methoxyacetanilide, *p*-hydroxyacetanilide, nitrobenzene, *p*-aminophenol, and *n*-butyric anhydride were obtained from Eastman Organic Chemicals, Rochester, N.Y. Derivatizing reagents iodomethane, iodoethane, *n*-iodopropane, *n*-iodobutane, *n*-iodoheptane, and tetramethylammonium hydroxide (TMH) (20% in methanol solution) were purchased from Aldrich Chemical Company, Inc., Milwaukee, Wis. *N*-Hydroxy-2-acetylaminofluorene was generously supplied to us by E. Weisburger, National Cancer Institute, Bethesda, Md.

Synthesis of Reference Compounds and Internal Standards. *N-Hydroxyacetanilide.* *N*-Hydroxyacetanilide was prepared by reduction of nitrobenzene to form phenylhydroxylamine as reported by Kamm (*18*). This compound was then acetylated with acetyl chloride in the presence of a sodium bicarbonate slurry as described by Priyadarshini and Tandon (*19*).

N-Hydroxy-p-alkoxyacetanilides. *N*-Hydroxy-*p*-methoxyacetanilide and *N*-hydroxy-*p*-ethoxyacetanilide were prepared similarly to those reported by Hinson (*3*). For example, to prepare *N*-hydroxy-*p*-methoxyacetanilide, zinc dust (0.4 mol) was added in one portion to a vigorously stirred reaction mixture containing 0.1 mol of *p*-nitroanisole, 0.12 mol of ammonium chloride, and 375 mL of water. After 15 min, the mixture was filtered, cooled to 5 °C, and then extracted with five 50-mL portions of diethyl ether. The ether extracts containing *N*-hydroxyanisidine were stirred in an ice bath at −5 °C and 40 mL of 4.2 M sodium bicarbonate was added. Freshly distilled acetyl chloride dissolved in anhydrous ether (1:25) was slowly added to the ether extracts. The disappearance of starting material was monitored by TLC on 250 μm silica gel. The TLC plates were developed in diethyl ether and sprayed with 5% ethanolic $FeCl_3$ for visualization. *N*-Hydroxyanisidine was observed as a purple spot (R_f 0.68) and *N*-hydroxy-*p*-methoxyacetanilide gave a wine colored spot (R_f 0.38). When no more starting material was detected by TLC, the reaction mixture was extracted with three 50-mL portions of 1.5 M ammonium hydroxide and the aqueous phase washed twice with dichloromethane. The alkaline solution was adjusted to pH 7.2 with concentrated formic acid and extracted three times with 50-mL portions of dichloromethane. The organic solution was washed with water and then evaporated.

p-Hydroxybutyranilide. *p*-Hydroxybutyranilide was prepared by reacting *p*-aminophenol with *n*-butyric anhydride. The procedure used was similar to that previously reported (*9*).

In Vitro Microsomal Reactions. Liver microsomal fractions were obtained from male golden Syrian hamsters (Charles River, Lakeside, N.J.) which were induced with 3-methylcholanthrene by I.P. injection (20 mg/kg in corn oil) every 24 h for 3 days. The microsomal fraction was prepared and the protein concentration

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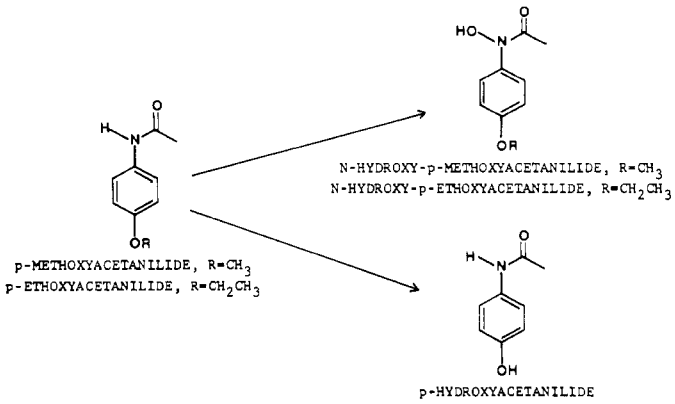

Figure 1. In vitro formation of *O*-dealkylated and *N*-hydroxylated metabolites from methacetin and phenacetin

determined (*20*, *21*). In addition, the cytochrome P450 content of the microsomes was measured using the method of Omura and Sato (*22*). The incubation mixture for the metabolism of *p*-methoxyacetanilide and its analogues contained 2.5 mL of 0.2 M NaF in 0.1 M potassium hydrogen phosphate buffer (pH 7.5), 0.1 mL of 50 mM *p*-methoxyacetanilide in methanol, and varying amounts of microsomes in a total volume of 4.5 mL. The reaction was initiated with 0.5 mL of 20 mM NADPH and carried out at 37 °C (exposed to air) for 10 min. Microsomal blanks were prepared for analysis by placing the incubation mixture in 5 mL of ethylacetate before adding the NADPH.

Isolation and Derivatization of Enzymatic Products. The microsomal reaction was terminated by transferring the reaction mixture to a 15-mL centrifuge tube (kept on ice) containing 5 mL of ethyl acetate to which was added a saturating amount of sodium chloride and the internal standards, *N*-hydroxyacetanilide (300 ng) and *p*-hydroxybutyranilide (30 μg). The mixture was shaken, centrifuged at approximately 1000 × g for 3 min and the organic layer transferred to a 10-mL pear-shaped flask. The aqueous phase was extracted with one additional 5-mL portion of ethyl acetate and was combined with the first extract. The combined extract was evaporated to dryness on a Rotovap at 35 °C. The residue was dissolved in 0.64 mL of acetone and 0.16 mL of 2.0% methanolic TMH. Acetone served equally as well as dimethyl sulfoxide or *N*,*N*-dimethylacetamide, reported by others (*23*, *24*), for a derivatization solvent and was easily removed by evaporation. After thorough mixing, 0.40 mL of the appropriate alkyl iodide was added, the solution mixed, and allowed to stand at room temperature for 30 min. The tetramethylammonium iodide formed was removed by centrifugation after adding 5 mL of diethyl ether and the supernatant was transferred to a 10-mL pear-shaped flask and evaporated to dryness on a Rotovap. The residue was dissolved in 100 μL of tetrahydrofuran and 5 μL injected into the gas chromatograph/mass spectrometer for quantitation.

Instrumentation. Quantitation of the enzymatic metabolites was performed on a Finnigan model 3200E gas chromatograph/mass spectrometer equipped with a chemical ionization source (CI) and a programmable multiple ion monitor (PROMIM). The output from the PROMIM was recorded on a Rikadenki model KA-40 four-pen recorder. In each case the protonated-molecular ion (M + 1) was monitored and peak heights were used for quantitation. The GC was fitted with a 1 mm i.d. × 0.35 m long glass column packed with 80/100 mesh Chromasorb G-HP coated with 3% XE-60, Chemical Research Services, Addison, Ill. The flow of methane carrier gas, also used as the reagent gas for chemical ionization, was 9 mL/min. The injector port temperature was 220 °C, transfer line 250 °C, and ion source approximately 60 °C. The column temperature was programmed from 110–200 °C at 8 °C/min. The operating pressure of the ion source was 1 Torr of methane and of the analyzer 2×10^{-5} Torr. An ionizing energy of 150 eV was used to produce the reactant ions. This same system was used to obtain conventional CI mass spectra of the reference compounds and enzymic products. The electron impact (EI) spectra were recorded with a Finnigan model 3000 gas chromatograph/mass spectrometer. Both instruments were interfaced to a System/150 data system (System Industries, Sunnyvale, Calif.). The nuclear magnetic resonance (NMR) spectra for structure determination of commercial and synthetic products were recorded on a Varian Model CFT-20 instrument operating at 80 MHz. The spectra were recorded in $CDCl_3$ (100% isotopic purity) or CD_3COCD_3 (100% isotopic purity) with tetramethylsilane (TMS) as the internal reference.

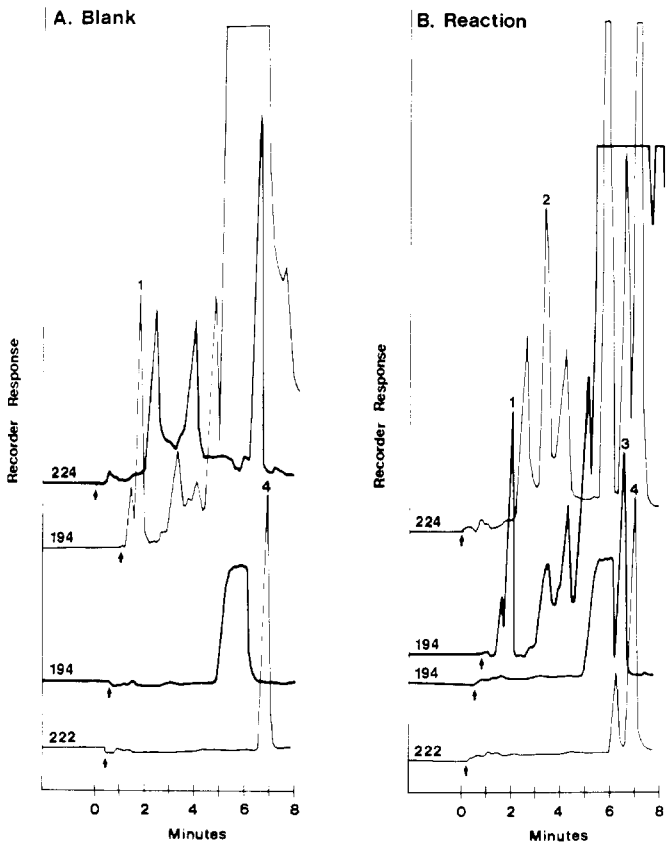

Figure 2. PROMIM recordings of (*A*) a microsomal blank containing the internal standard (1) *N*-(*n*-propyloxy)acetanilide (*m*/*z* 194) and (4) *p*-(*n*-propyloxy)butyramide (*m*/*z* 222). (*B*) a microsomal reaction containing (2) *N*-(*n*-propyloxy)-*p*-methoxyacetanilide (*m*/*z* 224), (3) *p*-(*n*-propyloxy)acetanilide (*m*/*z* 194), and their internal standards (1) *N*-(*n*-propyloxy)acetanilide (*m*/*z* 194), and (4) *p*-(*n*-propyloxy)butyranilide (*m*/*z* 222), respectively. The pens are offset for ease of recording; therefore arrows indicate actual sample injection time for each ion monitored. The ion at *m*/*z* 224 corresponds with 0 min on the abscissa. The large saturated signal observed for *m*/*z* 194 is due to the substrate, *p*-methoxyacetanilide

Quantitation of *N*-Hydroxy-*p*-methoxyacetanilide and *p*-Hydroxyacetanilide. Standard curves were generated using the isolation and derivatization procedure detailed above by adding known concentrations of reference *N*-hydroxy-*p*-methoxyacetanilide and *p*-hydroxyacetanilide to the incubation mixture (including microsomes and substrate) but without addition of NADPH. The standard curves were obtained using *n*-propyl iodide as the *O*-alkylating reagent. The compounds were analyzed as *N*-(*n*-propyloxy)-*p*-methoxyacetanilide and *p*-(*n*-propyloxy)acetanilide. The internal standard derivatives were *N*-(*n*-propyloxy)-acetanilide and *p*-(*n*-propyloxy)butyranilide, respectively. Typical PROMIM recordings of an extract from the microsomal reaction and a microsomal blank are shown in Figure 2.

RESULTS AND DISCUSSION

Identification of *N*-Hydroxy-*p*-methoxyacetanilide. The identification of *N*-hydroxy-*p*-methoxyacetanilide was verified by EI and CI mass spectrometry, and NMR spectroscopy. The EI spectrum (Figure 3) showed a molecular ion at *m*/*z* 181 and major fragments at *m*/*z* 165, *m*/*z* 139, and *m*/*z* 122. These ions correspond to losses of O, $COCH_2$, and O + $COCH_3$, respectively. With the exception of the ion observed at *m*/*z* 122, the fragmentation pattern agreed with

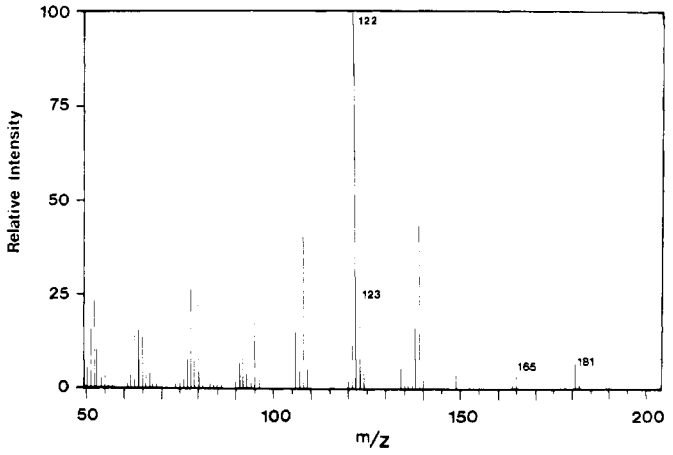

Figure 3. Electron impact mass spectra of synthesized *N*-hydroxy-*p*-methoxyacetanilide

those reported in the literature for the analogous compounds, *N*-hydroxyphenacetin (*3*), *N*-hydroxy-2-acetylaminofluorene (*25*), and *N*-hydroxy-*p*-chloroacetanilide (*12*). The CI mass spectrum showed a (M + 1) ion at m/z 182 and a major fragment ion at m/z 164 indicating loss of water in agreement with Hinson et al. (*3*). As further proof of structure of *N*-hydroxy-*p*-methoxyacetanilide, ^{1}H-NMR data obtained in $CDCl_3$ with reference TMS showed two singlets both of equal area indicating three protons at 2.02 and 3.82 ppm and were assigned to the *N*-acetyl and *p*-methoxy protons, respectively. A broad singlet corresponding to the *N*-hydroxyl proton was observed at 8.78 ppm and the four phenyl protons appeared as two sets of doublets in a typical A_2B_2 pattern at 6.90 ppm and 7.27 ppm. The *N*-hydroxy group on the acetanilides causes changes in the chemical shifts of various proton signals so that even low level contamination by *p*-methoxyacetanilide is detectable by NMR. No *p*-methoxyacetanilide was detected in the ^{1}H-NMR spectrum of the reference *N*-hydroxy-*p*-methoxyacetanilide. An attempt was made to resolve the discrepancy concerning the loss of O + $COCH_3$ ($M^+ - 59$) in our EI spectrum instead of O + $COCH_2$ ($M^+ - 58$) as reported by other investigators (*3*, *12*, *25*). Spectra obtained on our instrument for *N*-hydroxy-2-acetylaminofluorene and *N*-hydroxyphenacetin also showed a loss of ($M^+ - 59$) instead of ($M^+ - 58$). The EI spectra of *p*-methoxyacetanilide and *p*-ethoxyacetanilide showed molecular ions at m/z 165 and m/z 179, respectively, with major fragment ions at ($M^+ - 42$). It appears likely that the ($M^+ - 58$) and most of the ($M^+ - 16$) ions in the spectra of the *N*-hydroxy compounds reported by others arise from contamination with the reduced para-substituted acetanilides. This interpretation is supported by the fact that in our spectra the $(M^+ - 16)/M^+$ ratio is considerably lower than those from literature data (*3*). To test this hypothesis, a small quantity of *N*-hydroxy-*p*-ethoxyacetanilide was kept at room temperature for 4 h before mass spectral analysis. An increase in the ion ratios, $(M^+ - 58)/M^+$ and $(M^+ - 16)/M^+$ was observed suggesting decomposition of the *N*-hydroxy compound in preference to the *p*-ethoxyacetanilide which was present in small quantity as a contaminant.

Identification of *N*-hydroxy-*p*-methoxyacetanilide from the reaction mixture was confirmed by derivatizing with propyliodide followed by GS/MS analysis. Figure 4 compares the CI mass spectrum of reference *N*-(*n*-propyloxy)-*p*-methoxyacetanilide and the product from a microsomal reaction. No attempt was made to identify *N*-hydroxy-*p*-hydroxyacetanilide which should be readily detected using this procedure. Since microsomal incubation times were short, i.e., 10 min, and conversion of the parent compounds to *p*-hydroxyacetanilide and *N*-hydroxy-*p*-methoxyacetanilide

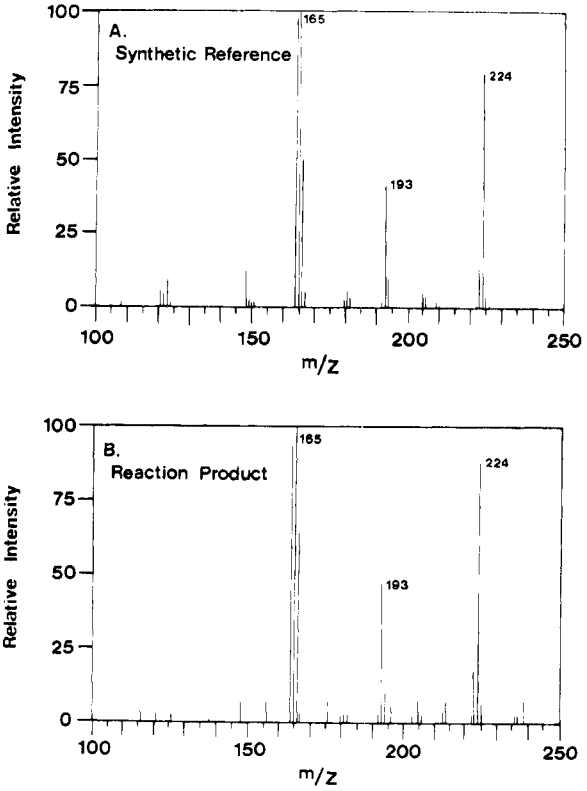

Figure 4. GC/MS CI mass spectra of (*A*) reference *N*-(*n*-propyloxy)-*p*-methoxyacetanilide, (*B*) extract from a microsomal reaction identifying approximately 40 ng of *N*-(*n*-propyloxy)-*p*-methoxyacetanilide

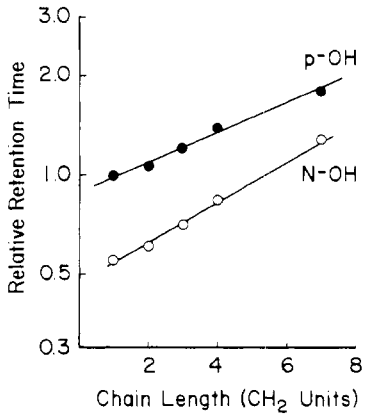

Figure 5. Log RR_t vs. methylene groups for methyl, ethyl, *n*-propyl, *n*-butyl, and *n*-heptyl derivatives of *N*-hydroxy-*p*-methoxyacetanilide and *p*-hydroxyacetanilide. All retention times were referenced to the *p*-methoxyacetanilide derivative

were 0–1.3% and 0–0.24%, respectively, it is unlikely enough product was available to serve as a substrate to form *N*-hydroxy-*p*-hydroxyacetanilide in sufficient quantities to be detected. In addition any *N*-hydroxy-*p*-hydroxyacetanilide formed would not be expected to interfere with our quantitation since its predicted gas chromatographic retention time from Figure 5 is greater than *N*-hydroxy-*p*-methoxyacetanilide and less than *p*-hydroxyacetanilide.

Quantitation of *N*-Hydroxy-*p*-methoxyacetanilide and *p*-Hydroxyacetanilide. The standards analyzed ranged from 0.3 μg/5 mL–0.2 μg/5 mL (15 ng–100 ng injected on column) for *N*-hydroxy-*p*-methoxyacetanilide and 20 μg/5 mL–100 μg/5 mL (1 μg–5 μg injected on column) for *p*-hydroxyacetanilide. The standard curves generated gave good linearity over the range of the analysis with correlation coefficients of 0.98 and 0.99 for *N*-hydroxy-*p*-methoxyacetanilide and *p*-hydroxyacetanilide, respectively. Relative standard deviations

were determined from multiple extractions of the lowest and highest reference standards. The results were 0.30 μg/5 mL ± 14.4% S.D. and 1.97 μg/5 mL ± 3.1% S.D. for *N*-hydroxy-*p*-methoxyacetanilide and 19.0 μg/5 mL ± 2.2% S.D. and 103 μg/5 mL ± 8.0% S.D. for *p*-hydroxyacetanilide. This analysis demonstrates the wide range of concentrations that can be quantitated simultaneously using the GC/MS technique of SIM.

Earlier attempts to quantiate these two enzymatic products using trimethylsilyl derivatives gave nonreproducible results due to the instability of the silylated derivatives. Our *O*-alkylated derivatives are stable and give good reproducibility upon repeated injections of the solution for up to one week when stored in tetrahydrofuran at −17 °C. Another distinct advantage of the *O*-alkylated derivatives is the variety of compounds that can be formed using essentially the same procedure. This can be quite important when attempting to develop a simple quantitative method for extracts from biological systems which contain numerous endogenous compounds which could result in interference. Figure 5 shows the relationship between chain length of the alkylating agent in methylene units and relative retention times for a series of *O*-alkyl derivatives and allows a systematic approach to establishing where the respective alkyl derivatives will occur in the chromatogram.

The procedure described here is expected to be general in nature and suitable for derivatization and quantitation of phenolic and *N*-hydroxyl metabolites. It is of special significance due to its suitability for quantitating the *N*-hydroxyl metabolites which are linked to various toxic reactions such as methemoglobinemia, hepatotoxicity, nephropathy, and even carcinogenesis. This is becoming even more relevant as the *N*-hydroxy compounds are found to be present as metabolites of common drugs in humans (i.e., amobarbital (*13*), meperidine (*26*), and acetaminophen (*27*)).

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