Introduction

Early determination of tissue pharmacokinetic parameters and metabolite identification plays an important role in the drug discovery and development processes. The implementation of new technologies, such as quantitative phosphor imaging of whole-body sections (QWBA) in ADME research has become the norm for discovery and mechanistic studies. This methodology provides detailed quantitative information on all tissues throughout the body. Furthermore, it can be easily coupled with metabolite profiling to reveal substantial details about the disposition and metabolism of the drug candidate.

Localization at the cellular level can be obtained by micro-autoradiography (MARG), which in itself is a photographic technique that offers both qualitative and semi-quantitative information.

The combination approach of QWBA, MARG, and tissue metabolite profiling using radio-HPLC and/or LC/MS/MS can reveal specific localization of the dosed compound and metabolites at different sites. This approach can be applied to multiple disciplines in drug discovery and development.

The study described in this application note demonstrates how QWBA and MARG were used to quantitatively examine whole-body tissue distribution and cellular localization; and to obtain some metabolism information of [^{14}C]-3′-Azido-2′-deoxythymidine (^{14}C-AZT) in fetal and maternal rat brain and liver. The data clearly show the different distribution of ^{14}C-AZT in various tissues after a single intravenous (IV) dose to pregnant rats.

Methods

In-Life

Four pregnant (gestation day 17) female Sprague Dawley rats were each given a single IV bolus 60 mg/kg dose of ^{14}C-AZT (3′ Azido 3′-deoxythymidine, [2-^{14}C]; radiochemical purity = 99.7%; 53 mCi/mmol; Moravek Biochemicals and Radiochemicals, Brea, CA) formulated in 0.9% sterile saline. Two of the rats were euthanized at approximately 90 minutes post-dose and their carcasses were immediately snap-frozen, processed, and analyzed using QWBA as outlined below. The two remaining rats were euthanized and necropsied to obtain maternal brain, liver, and fetuses, which were snap-frozen in liquid nitrogen for MARG processing and analysis.

QWBA Analysis

The frozen carcasses were embedded in 2% carboxymethylcellulose and each block was mounted on the object stage of a cryomicrotome (Leica CM3600 Cryomacrocut, Nussloch, Germany) maintained at approximately -20°C. Sagittal whole-body sections, at approximately 40 µm thickness, collected onto adhesive tape (Scotch Tape No. 8210, 3M Ltd., St. Paul, MN, USA), were taken from various levels of interest throughout each frozen embedded carcass until samples of the following tissues or biological matrices were obtained, where possible – fetal tissues: brain, liver, blood, eye, heart, kidney, adrenal, thymus, spinal cord, stomach, and lung; maternal tissues: adipose (brown and white), adrenal gland, amnion and fluid, bile (in duct), blood (cardiac), bone, bone marrow, brain (cerebrum, cerebellum and medulla), cecum (and contents), eye (uveal tract and lens), Harderian gland, cardiac muscle (heart), renal cortex and medulla, large intestine (and contents), liver, lung, lymph node, nasal turbinates, ovary, pancreas, pituitary gland, placenta, salivary gland, skeletal muscle, skin, stomach (gastric mucosa and contents), small intestine (and contents), spleen, spinal cord, thymus, thyroid gland, uterus, vagina, and urinary bladder (and contents).

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Methods continued

Whole-body sections were allowed to dry by sublimation in the cryomicrotome at -20°C for at least 48 hours and were then mounted on cardboard backing, covered with plastic wrap, and exposed along with ^{14}C-spiked blood calibration standards to ^{14}C-sensitive phosphor imaging plates (Molecular Dynamics / GE Healthcare, Piscataway, NJ). The imaging plates and sections were enclosed in exposure cassettes and allowed to expose at room temperature for four days. At the end of the exposure period, the sections were removed from the imaging plate. The plates were scanned and the digital images were stored onto a computer server using the Typhoon 9410, image acquisition system (Molecular Dynamics/GE Healthcare, Piscataway, NJ). Quantification of tissue concentrations, relative to the calibration standards, was performed by image densitometry using MCID image analysis software (GE Healthcare, Piscataway, NJ; formerly Imaging Research, St. Catherines, Ontario, Canada). The concentrations of radioactivity were expressed as the µg equivalents of AZT per gram of sample (µg equiv/g).

MARG Analysis

The frozen brain and liver samples obtained at necropsy were cryosectioned at 5 µm under darkroom conditions, and were collected onto glass slides that were pre-coated with photographic emulsion (Kodak NTB photographic emulsion). Slides were placed in sealed light-safe slide boxes and allowed to expose the photographic emulsion for 7 days before being developed, fixed and stained with hematoxylin and eosin. All slides were examined for cellular localization using light microscopy.

Metabolite Profiling

Samples of maternal and fetal liver and brain were collected from the two residual frozen animal carcasses used for QWBA. The tissues were homogenized in 20 mM sodium dodecyl sulfate, and the homogenate was extracted with acetonitrile. After centrifugation at 3,000 RPM for 5 minutes, the supernatant was transferred to a new tube, and the pellet was extracted one more time. The supernatant was combined, evaporated under a nitrogen stream, and the residue was reconstituted in water/acetonitrile (90:10). The system for metabolite profiling consisted of a LEAP HTC PAL autosampler, two Shimadzu HPLC pumps, and a β-RAM Model 3 radio flow-through detector. The radio flow-through detector was controlled by Laura Lite 3 software. AZT and its metabolites were separated on a reverse phase column (Sepax HP C18, 4.6 x 250 mm), which was eluted with 10 mM ammonium formate/0.1% formic acid (Solvent A) and acetonitrile/0.1% formic acid (Solvent B) using a gradient from 2% B to 22% B in 20 minutes at a flow rate of 0.8 mL/minute.

Results and Discussion

Figures 1 and 2 are representative whole-body autoradioluminographs. Maternal and fetal tissue concentration data obtained by QWBA showed clear differences in maternal and fetal tissue distribution (Tables 1 & 2). Fetal brain ^{14}C-AZT concentrations (0.159 µg equiv/g) were 8.5-fold higher than maternal brain (0.018 µg equiv/g). Fetal liver concentrations were also notably higher than maternal liver (0.507 µg equiv/g and 0.318 µg equiv/g, respectively). In most other fetal and maternal tissues, the concentrations were similar. However, concentrations in the fetal kidney (0.205 µg equiv/g) were substantially lower than concentrations in maternal kidney (~1.0 µg equiv/g).

The observed differences between the fetal and maternal in brain, liver, and kidney concentration suggested that these fetal and maternal tissues were being exposed to either varying amounts of the parent drug, ^{14}C-AZT, and/or the metabolites. Therefore the efficacy of the drug in maternal versus fetal tissue may be substantially different.

The differences in liver and kidney concentrations also suggested that ^{14}C-AZT was being absorbed, metabolized, and/or eliminated differently in fetus and mother. This was most likely due to underdeveloped biochemical pathways in fetal organs and/or an underdeveloped anatomy. Additional metabolite profiling of the fetal and maternal tissues is necessary to distinguish the identity of the radiolabel to determine the actual exposure to AZT and/or its metabolites. Nevertheless, the striking differences in distribution are apparent. This provides an opportunity for further consideration if additional mechanistic studies are needed to move a candidate into development.

MARG data provided a microscopic evaluation of the distribution in these maternal and fetal tissues. MARG showed that the cellular distribution of the drug-derived radioactivity was different in both maternal and fetal brain and liver (Figure 3). This in turn suggested there may be differences in tissue effects. Distribution in the fetal brain was more diffusely distributed and at a higher concentration than that seen in the maternal brain. The fetal brain had high amounts of label material throughout all out all regions, thus higher and possible over-exposure to the test article. The pia mater of the maternal choroid plexus (not shown) was also high and was probably due to the presence of transporters for ^{14}C-AZT, which were absent in the fetal brain. The blood-brain-barrier in the fetal brain at day 17 of gestation is commonly thought to be underdeveloped and thus probably accounted for the higher amounts of radioactivity.

Radiochromatograms of brain extracts (Figure 4) revealed that the higher concentration in fetal brain was most likely due to a higher proportion of the AZT-glucuronide metabolite and not parent drug, whereas the proportion of parent drug was higher in the maternal brain. MARG in the fetal liver also showed denser and more evenly distributed label material than in the maternal liver. Localization in the adult liver was centered near the central vein, bile canaliculi, and sinusoidal spaces suggesting the elimination pathway through biliary excretion. The whole-body autoradioluminographs supported this notion as drug-derived radioactivity was apparent in the lumen of the maternal gastrointestinal tract, but was not evident in the fetal GI tract. Once again, underdevelopment of the fetal liver may account for a slower elimination of ^{14}C-AZT derived radioactivity and may be due to the presence of metabolites and/or the parent drug. Radiochromatograms supported this theory as no parent AZT was detected in maternal liver but there were 3 metabolites present, whereas fetal liver primarily showed the presence of parent AZT and AZT-glucuronide (Figure 4). A previous study showed that ~11% of administered IV dose to adult rats was present in bile as 5'-O-glucoronide-AZT; ~80% was in urine as AZT; and ~10% was unknown (J Pharmacol Exp Ther. 1991, Dec, 259(3):1261-70). The work described in this Application Note extends those findings and presents new data on fetal exposure.

Summary

This data demonstrated that an innovative study design using a combination of QWBA, MARG, and metabolite profiling can provide excellent data on quantifiable tissue and cellular localization. These types of study designs can also positively identify the parent drug and/or metabolites associated with the radioactivity imaged.

These techniques can be executed using low level, non-penetrating radioactivity over long periods of time, and offers a wealth of information from a single study. This Application Note demonstrates how this type of study with only a few animals can provide an enormous amount of information when conducted early in drug discovery or development. This information can help identify potential liabilities and/or additional therapeutic targets for drugs.

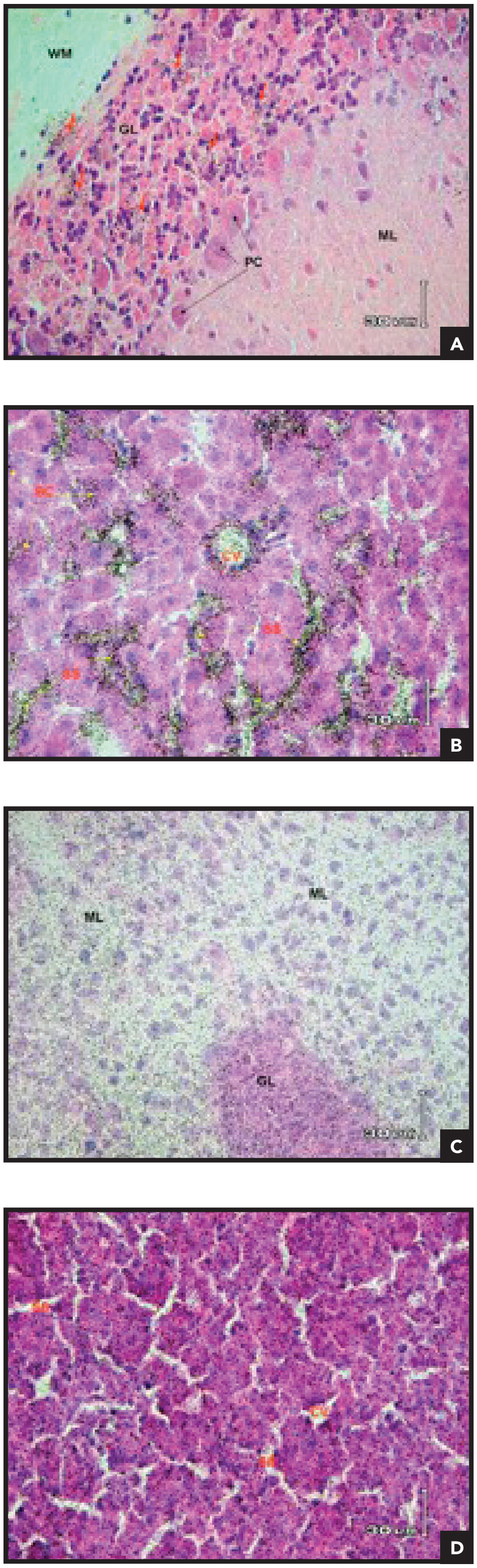

Figure 3. *Photomicroautoradiographs of the cellular localization of ^{14}C-AZT-derived radioactivity in the brain and liver of a pregnant rat (A and B respectively) and in the brain and liver of a 17-day old fetus (C and D respectively).*

(Hematoxylin & Eosin Stain, 400X; ML = Molecular Layer, GL = Granular Layer, WM = White Matter, PC = Purkinje Cells)

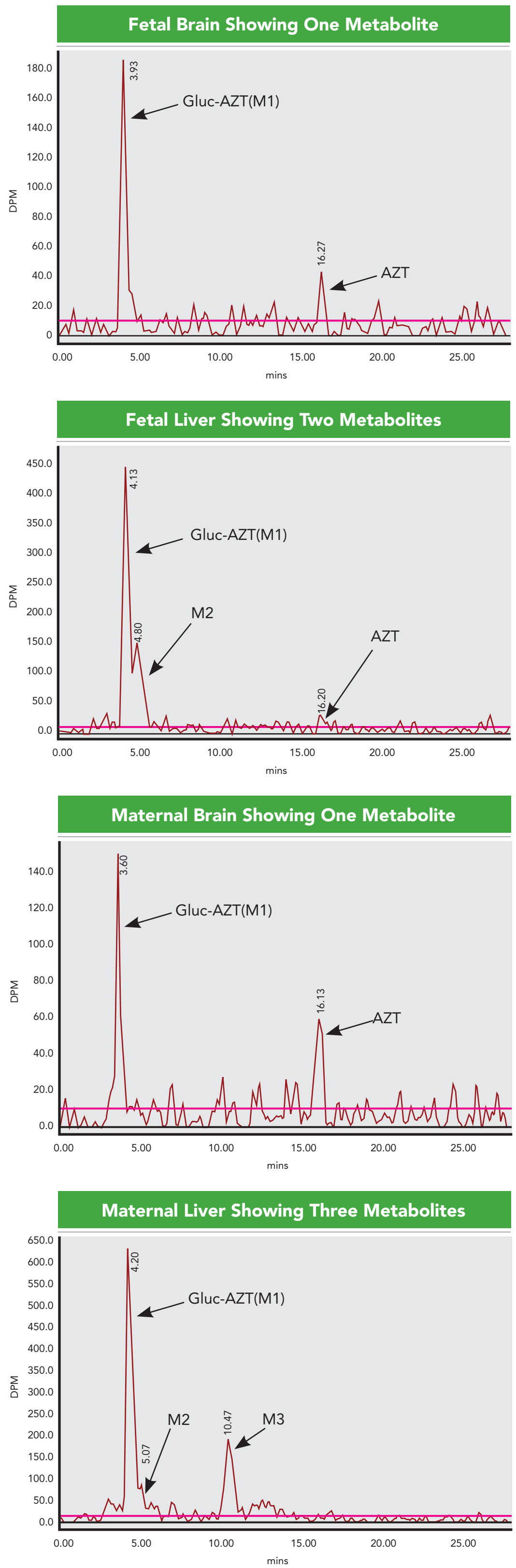

Figure 4. *Radiochromatograms showing the metabolite profiles obtained from maternal and fetal liver and brain samples after a single intravenous administration of ^{14}C-AZT.*

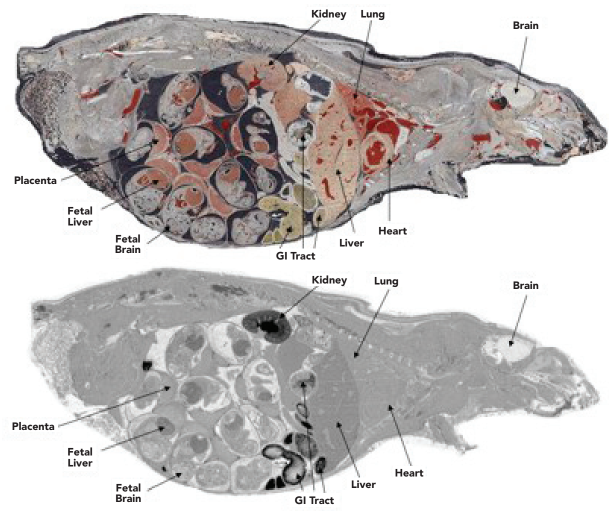

Figure 1. *Whole-Body Section and the resulting Autoradioluminograph of a pregnant rat 90 minutes after an IV administration of ^{14}C-AZT (60 mg/kg)*

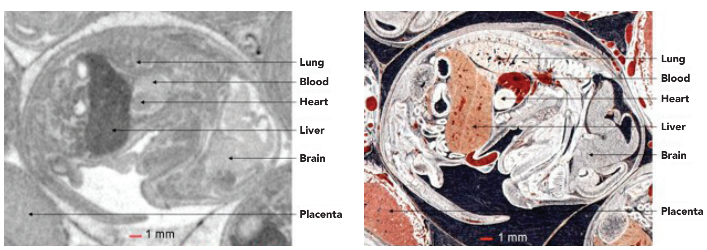

Figure 2. *Whole-Body Section and resulting Autoradioluminograph of a fetal rat 90 minutes after an IV administration of ^{14}C-AZT (60 mg/kg)*

Concentration (µg equivalent / g tissue)

Tissue	Maternal Rat #1	Maternal Rat #2	Mean
Placenta	0.263	0.191	0.227
Amnion	0.112	0.125	0.119
Amniotic fluid	0.031	0.027	0.029
Brain	0.164	0.153	0.159
Liver	0.596	0.417	0.507
Blood	0.176	0.175	0.176
Eye	0.265	0.300	0.283
Heart	0.229	0.227	0.228
Kidney	0.241	0.169	0.205
Adrenal Gland	0.280	0.293	0.287
Thymus	0.401	0.353	0.377
Spinal Cord	0.167	0.152	0.160
Stomach	0.326	0.078	0.202
Lung	0.330	0.284	0.307

LLOQ = 0.00084 µCi/g / 0.1984 µCi/µg = 0.004 µg equiv/g tissue
ULOQ = 8.177 µCi/g / 0.1984 µCi/µg = 41.2 µg equiv/g tissue

Table 1. *Concentration of ^{14}C-AZT Equivalents in Fetal Tissues 90 minutes After IV Administration at 60 mg/kg*

Concentration (μg equivalent / g tissue)				
Tissue Type	**Tissue**	**Maternal Rat #1**	**Maternal Rat #2**	**Mean**
Vascular/ Lymphatic	Blood (cardiac)	0.283	0.184	0.234
	Bone Marrow	0.663	0.385	0.524
	Lymph Nodes	0.270	0.221	0.246
	Spleen	0.517	0.203	0.360
	Thymus	0.315	0.191	0.253
Excretory/ Metabolic	Bile (in duct)	0.638	1.510	1.074
	Renal Cortex	0.926	1.078	1.002
	Renal Medulla	1.378	1.344	1.361
	Liver	0.371	0.264	0.318
	Urinary Bladder	0.405	0.237	0.321
	Urinary Bladder (contents)	47.140	14.195	30.668
Central Nervous System	Brain	0.021	0.015	0.018
	Spinal Cord	0.021	0.016	0.019
Endocrine	Adrenal Gland	0.266	0.192	0.229
	Pituitary Gland	0.281	0.220	0.251
	Thyroid Gland	0.279	0.193	0.236
Secretory	Harderian Gland	0.162	0.107	0.135
	Pancreas	0.272	0.208	0.240
	Salivary Gland	0.286	0.178	0.232
Adipose	Adipose (brown)	0.199	0.108	0.154
	Adipose (white)	0.005	0.009	0.007
Dermal	Skin	0.222	0.146	0.184
	Ovary	0.245	0.143	0.194
	Uterus	0.278	0.184	0.231
Reproductive	Mammary Gland	0.142	0.103	0.123
	Vagina	0.296	0.195	0.246
Skeletal/ Muscular	Bone	0.011	0.018	0.015
	Heart	0.302	0.183	0.243
	Skeletal Muscle	0.294	0.169	0.232
Respiratory Tract	Lung	0.278	0.191	0.235
	Nasal Turbinates	0.130	0.085	0.108
Alimentary Canal	Large Intestine	0.316	0.246	0.281
	Large Intestine (contents)	0.031	0.241	0.136
	Stomach (gastric mucosa)	0.279	0.189	0.234
	Stomach (contents)	0.510	0.374	0.442
	Small Intestine	0.553	0.524	0.539
	Small Intestine (contents)	2.794	9.873	6.334
Ocular	Eye Uveal Tract	0.155	0.090	0.123
	Eye Lens	0.022	0.015	0.019

Lower Limit Of Quantification (LLOQ) = 0.00084 μCi/g / 0.1984 μCi/μg = 0.004 μg equivalent / g tissue
Upper Limit Of Quantification (ULOQ) = 8.177 μCi/g / 0.1984 μCi/μg = 41.2 μg equivalent / g tissue

Table 2. *Concentration of 14C-AZT Equivqlents in Maternal Tissues 90 minutes After IV Administration at 60 mg/kg*

Delaware Technology Park
3 Innovation Way, Suite 240
Newark, DE 19711

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TEL (302) 369-5601
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