

Comparative *in vitro* Metabolism of Benzyl Amine Containing Test Drug Using Human, Rat, and Dog Plasma – Investigation of Amine Oxidase Involvement in Drug Metabolism



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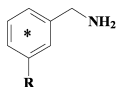
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Abstract

Poor extractability of drug-related compounds from plasma is a possible indication of the formation of reactive metabolites, resulting in covalent binding with the endogenous matrix, which in turn may become a potential cause of adverse reactions or organ toxicity. The biotransformation of a radiolabeled test article containing a benzylamine moiety (^{14}C)-TA) was investigated *in vitro* using dog, human, and rat plasma. The incubation mixtures were analyzed by HPLC/radio-detection/MS. Amine oxidase involvement in the metabolism of TA in plasma was investigated by using specific amine oxidase inhibitors. Metabolism of TA via oxidative deamination was rapid in dog plasma, but TA was stable in human and rat plasma. Among the four different amine oxidases evaluated, semicarbazide sensitive amine oxidase was responsible for the metabolism in dog plasma. In addition, dog plasma yielded a significant amount of unextractable radioactivity in the post extraction pellets while rat and human plasma yielded appropriate extraction efficiencies, demonstrating that the metabolites in dog plasma may have been strongly bound to plasma protein. Possible permanent covalent binding of TA-related compounds to plasma protein was investigated by incubation of ^{14}C -TA with denatured plasma as well as by using Western blot assay. The binding of the unextractable radioactivity to plasma protein in the pellets was shown to be reversible by Western blot assay. However, the different metabolite profile in dog plasma indicated that dogs may not be an appropriate toxicity model for the TA development.

Introduction

The structure of TA is shown below:



*The position of ^{14}C -label

The objectives of the study were to investigate (1) the *in vitro* biotransformation profiles of ^{14}C TA in plasma from rats, dogs, and humans; (2) the responsible enzymes for TA metabolism in plasma; (3) the possibility of covalent binding of TA metabolites to plasma proteins

Materials and Methods

Test System: (1) Pooled plasma from male Beagle dogs, male Sprague Dawley rats, and male humans

Concentration: 10 μM of test article (^{14}C TA)

Incubations:

a. Plasma; 4 hr at 37 °C. Sampling at 0.5, 1, and 4 hr.

b. Plasma with Amine Oxidase Inhibitors; 1 hr at 37 °C. A list of amine oxidases is shown in Table 1.

c. Heat-denatured plasma (plasma heated at 70 °C for 1 hr, followed by cooling and sonication prior to incubation with ^{14}C TA for 4 hr at 37 °C.

Name	Concentration
clorgyline	~0.5 mM
pargyline	~0.5 mM
semicarbazide	~20 μM
hydralazine	~0.5 mM

Table 1. A list of Amine Oxidase Inhibitors Used for the Study

Sample Processing: The incubation mixture was extracted with methanol (1:2, v/v), vortexed, and centrifuged. The extraction step was repeated with a mixture of water:methanol (1:2, v/v). The supernatant was analyzed by HPLC/RAM or LC/MS.

HPLC System: Waters Model 2695

Column: Ace 3 C18, 4.6 x 150 mm, 3 μm

Mobile Phase: A: 0.02M $\text{CH}_3\text{COONH}_4$ (pH = 4 with HCOOH); B: CH_3OH

Radiochromatograms: Fractions were collected into DeepWell LumaPlate™-96 plates and the collected fractions were dried, and counted using a Packard TopCount® NXT™ solid scintillation counter. Reconstructed HPLC chromatograms were prepared using the determined radioactivity in each fraction.

Mass Spectrometer: Finnigan MAT TSQ-7000

High Performance Quadrupole Mass Spectrometer

Ionization Modes: Positive electrospray

Ion Spray (IS): 4.5 KV

Capillary Temperature: 230 °C

Visualization of Radioactive Spots from Western Blot Assay: FUJIFILM Bioanalyzing Systems (BAS) 1800II Phosphorimager

Visualization of Separated Proteins from Western Blot Assay: The separated proteins were

visualized by immersing the nitrocellulose membrane in SimplyBlue™ SafeStain, and the MW's of the proteins in the resulting precipitates were assessed by comparison to reference protein molecules that were analyzed alongside.

Methods (Continued)

ANALYSES OF BOUND RESIDUES IN PLASMA INCUBATION MIXTURES

Protease Hydrolysis of Bound Residues: Post extraction solids (PES) from dog and human plasma were subjected to protease hydrolysis (from bovine pancreas)

Western Blot Assay: PES from dog plasma incubate was resuspended in water. It was subjected to Western Blot assay alongside a mixture of reference proteins. Prior to loading, the protein in the suspension was denatured. Native protein and the supernatant from the initial extraction were also loaded as reference.

Electrophoresis; NuPAGE® Novex Tris-Acetate Native gels, or NuPAGE® Novex Bis-Tris gels with MOPS or MES SDS running buffer were used for each corresponding electrophoresis run.

Results

Species	% Extractable	% in Pellets
Rat	88%	12%
Dog	61%	39%
Human	79%	21%

Table 2. Percent Distribution of Radioactivity after Incubation in Plasma for 4 Hours

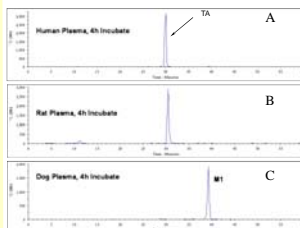


Figure 1. Metabolite Profiles of TA in Human (A), Rat (B), and Dog Plasma (C)

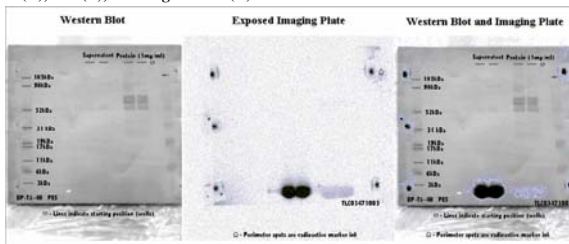


Figure 4. Western Blot of Dog Plasma Pellets (Denatured/Reduced Protein; MW Range of 185K to 3K Da)

Results (Continued)

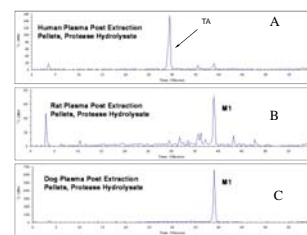


Figure 2. Metabolite Profiles of Protease Hydrolysate of the Post Extraction Pellets from Human (A), Rat (B), and Dog Plasma Incubates (C)

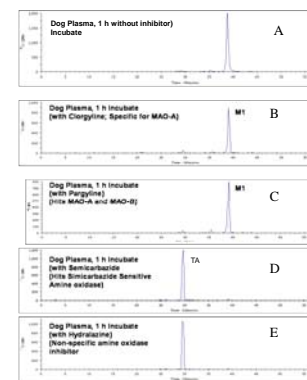


Figure 3. Metabolite Profiles of TA in Dog Plasma Incubates in the Presence of Monoamine Oxidase Inhibitors; (A) without inhibitor (B) Clorgyline (C) Pargyline (D) Semicarbazide (E) Hydrazine

Results and Discussion

TA was stable in human and rat plasma (Figure 1). It was unstable in dog plasma, yielding an aldehyde metabolite (M1) as the major metabolite. Dog plasma incubate also yielded a significant amount of unextractable radioactivity in the post extraction pellets (Table 2). The extent of radioactivity remaining in the pellets was more significant when the aldehyde metabolite was a major metabolite in the plasma.

Protease hydrolysis of the pellets released the entire radioactivity to the supernatant. The majority of the released radioactivity from dog plasma pellets was M1 (Figure 2).

Incubation of dog plasma with four different amine oxidase inhibitors demonstrated that semicarbazide sensitive amine oxidase was responsible for the metabolism of TA to yield the aldehyde metabolite in dog plasma (Figure 3).

The denaturation of dog plasma protein prior to incubation with ^{14}C TA yielded the majority of the radioactive residues in the incubation mixture extractable to the supernatant. In addition, the Western blot assay of the pellets demonstrated that the bound residues were reversible (Figure 4) rather than irreversibly bound to proteins. The results show that the radioactive residues in the supernatant as well as protein matrix moved to the bottom of the gel along with the proteins of MW ~3k Da and smaller, indicating that the radioactive residues in the pellets were completely released from the protein matrix and moved as free molecules rather than as protein bound molecules.

Conclusion

Extensive analyses in dog plasma showed that binding of unextractable TA-related radioactivity in pellets was reversible. However, the different metabolite profile in dog plasma indicated that dogs may not be an appropriate toxicity model for the TA development.