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Metabolic activation of Pioglitazone identified from rat and human liver microsomes and freshly isolated hepatocytes

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Running Title Page

A) Metabolic activation of Pioglitazone

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Non-standard Abbreviations:

LC/MS	High performance liquid chromatography with tandem mass spectrometry
GSH	Reduced glutathione
SPE	Solid phase extraction
MCX	Mixed-mode cation exchange
Q-ToF	Quadrupole time of flight
MRM	Multiple Reaction Monitoring

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Abstract

Pioglitazone is in the class of compounds known as the thiazolidinediones and is used to treat type 2 diabetes mellitus. The first in its class compound, troglitazone, was withdrawn from the U.S. market in 2000 due to a high incidence of hepatotoxicity and drug-induced liver failure. Reactive ring-opened products of troglitazone have been identified and evidence suggests that these reactive intermediates might be a potential cause of hepatotoxicity. The present work shows that pioglitazone has a reactive ring-opened product which was trapped by glutathione and positively identified by LC/MS accurate mass measurements. The novel thiazolidinedione ring-opened products of pioglitazone were identified in rat and human liver microsomes and in freshly isolated rat but not human hepatocytes.

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Pioglitazone is in the class of compounds known as the thiazolidinediones (troglitazone and rosiglitazone are in this class) and is used to treat type 2 diabetes mellitus by targeting the peroxisome proliferator-activated receptor-gamma (PPAR γ). Pioglitazone is metabolized in the liver primarily by Cytochromes P450 (CYPs) 3A4 and 2C8 (Insert 2002; Sahi, Black et al. 2003) to several oxidative metabolites (Krieter, Colletti et al. 1994; Sohda, Ikeda et al. 1995; Tanis, Parker et al. 1996; Kiyota, Kondo et al. 1997; Eckland 2000; Shen, Reed et al. 2003). There have been extensive reports published on the hepatotoxic effect of troglitazone that led to its removal from the U.S. market in 2000 (Gitlin, Julie et al. 1998; Neuschwander-Tetri, Isley et al. 1998; Watkins and Whitcomb 1998; Faich and Moseley 2001). Evidence suggests that a potential cause of hepatotoxicity by troglitazone might be the formation of a reactive metabolite that could bind to cellular protein or DNA (Kassahun, Pearson et al. 2001; Smith 2003; Tolman and Chandramouli 2003). The glitazones (figure 1) all have a thiazolidinedione ring and at least one of the metabolites of troglitazone, pioglitazone, and rosiglitazone involves a ring-opened product of the thiazolidinedione ring (Kassahun, Pearson et al. 2001; Shen, Reed et al. 2003; Alvarez-Sanchez and Paehler 2004). However, the ring-opened product of pioglitazone has only been identified in dog liver microsomes. The ring-opened products for rosiglitazone (Avandia®), currently the number one prescribed medicine in its class, and pioglitazone have yet to be discovered or reported as being formed in human *in vitro* systems or in the clinic. Due to recent findings of liver injury in patients undergoing pioglitazone treatment (Maeda 2001; Chase and Yarze 2002; May, Lefkowitz et al. 2002), pioglitazone was evaluated in rat and human liver microsomes and suspensions of freshly isolated hepatocytes to determine if a reactive ring-opened product would be formed. To accomplish this a generic method for trapping reactive electrophilic metabolites was utilized (Chen, Zhang et al. 2001). In this method, the reactive intermediate is trapped using excess glutathione (GSH) and the trapped adduct is detected using the characteristic 129 amu neutral loss from GSH. This method offers an advantage of having the potential to trap and detect all species reactive with GSH and providing molecular weight information. The molecular weight information was used to further identify metabolites by quadrupole time of flight (Q-ToF) accurate mass measurements. Shen and colleagues (Shen, Reed et al. 2003) first identified a thiazolidinedione ring-opened product (M-X) of pioglitazone in dog liver microsomes. However, they did not observe such a metabolite following incubations in rat or human liver microsomes. In this report, we have identified the thiazolidinedione ring-opened product (M-X) of pioglitazone in rat and human liver microsomes and two novel ring-opened glutathione conjugates of pioglitazone in

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rat and human liver microsomes and suspensions of freshly isolated rat hepatocytes. None of the ring-opened products were positively identified in freshly isolated human hepatocytes.

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Materials and methods

Materials. Pioglitazone was synthesized by the Medicinal Chemistry department at GlaxoSmithKline (GSK). The structure of pioglitazone is shown in Figure 1 (MW = 356.45). Potassium phosphate monobasic (KH_2PO_4) and potassium phosphate dibasic trihydrate ($\text{K}_2\text{HPO}_4 \bullet 3\text{H}_2\text{O}$) were purchased from J.T. Baker (Phillipsburg, NJ). Magnesium chloride (MgCl_2), β -nicotinamide adenine dinucleotide phosphate, reduced (NADPH), dexamethasone, Percoll[®], and glutathione (GSH) were purchased from Sigma (St. Louis, MO). Rat (RLM) and human liver microsomes (HLM) were purchased from XenoTech, LLC (Lenexa, KS). Human hepatocytes were purchased from In Vitro Technologies (Baltimore, MD) and XenoTech, LLC (Lenexa, KS) and rat hepatocytes were isolated at GSK from male SD rats purchased from Charles River Laboratories using the method described below. Fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium (DMEM) were purchased from Cambrex Biosciences (Walkersville, MD). Penicillin, streptomycin, and insulin were purchased from Gibco (NY) and ITS+ culture supplement (625 $\mu\text{g}/\text{ml}$ insulin, 625 $\mu\text{g}/\text{ml}$ transferrin, 625 ng/ml selenium, and 100 mg/ml albumin) was purchased from BD Biosciences (Bedford, MA).

Microsomal Incubation. Reagents were prepared by dissolving solids or diluting liquids in potassium phosphate buffer (50 mM) at concentrations shown below. A 10 mM stock solution of pioglitazone was prepared in dimethyl sulfoxide (DMSO). Each incubation vial contained substrate (100 μM pioglitazone final concentration, 1% DMSO in incubate), RLM or HLM-Buffer (345 μL of a 20 mg/mL stock preparation, 2 mg/mL liver microsomes), GSH (10 mM final concentration, no GSH controls received 100 μL of buffer), and cofactor (2 mM NADPH and 5 mM MgCl_2 final concentration, no cofactor controls received 50 μL buffer) in this order. Three separate control incubations were performed: incubation in the absence of substrate, glutathione, or cofactor. The final incubation volume was 500 μL for the glutathione-trapping experiment and scaled up to 5 mL for LC/MS accurate mass measurement. Samples were incubated at 37°C for 30 min then quenched with 2% trifluoroacetic acid (TFA, 500 μL) or 0.1% formic acid in acetonitrile (5mL).

Rat Hepatocyte Isolation. Rat hepatocytes were isolated from male SD rats using a modification of the two-step collagenase digestion method described by Seglen (Seglen 1976). Rat livers were first perfused with a calcium-free buffer containing glucose and EDTA and then with buffer containing calcium and collagenase (both for 10 min at a flow rate of $\sim 30 \text{ mL}/\text{min}$). Hepatocytes were dispersed from the digested liver in DMEM supplemented with 5% FBS, 50 U/mL penicillin, 50 $\mu\text{g}/\text{mL}$ streptomycin, 4 $\mu\text{g}/\text{mL}$ insulin, and 1 μM dexamethasone, then washed

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by low-speed centrifugation (50g for 3 min). The cell pellet was resuspended in a 1:1 mixture of 90% isotonic Percoll[®] and supplemented DMEM, then centrifuged (70g for 5 min). After performing a final wash, cells were resuspended in serum-free DMEM containing 0.1 μ M dexamethasone, 50 U/mL penicillin, 50 μ g/mL streptomycin, and ITS+ (625 μ g/ml insulin, 625 μ g/ml transferrin, 625 ng/ml selenium, and 100 mg/ml albumin). Viability of hepatocytes was 86% at the start of the incubation as determined using trypan blue.

Human Hepatocyte Preparation. Freshly isolated human hepatocytes were purchased from In Vitro Technologies and recovered from the shipping media as per vendor's instructions. Viability of the hepatocytes was 87% at the start of the incubation as determined by trypan blue exclusion. Cryopreserved human hepatocytes were purchased from In Vitro Technologies (individual donor) and XenoTech, LLC (three donors) and thawed as recommended by the vendor prior to use in incubations. Cells from three different donors (XenoTech) were thawed individually and pooled prior to incubation (initial viability was 83%, 83% and 90%). Viability of cryopreserved hepatocytes from In Vitro Technologies was at least 70% at the start of the incubation.

Hepatocyte Incubation. For incubations, rat and human hepatocytes were diluted in serum-free DMEM (supplemented as described above) to 1.0×10^6 cells/mL and 0.5 mL was added per well in 12-well plates. Hepatocytes were then placed on a rotary shaker at 70 rpm in a humidified incubator (95/5% air/CO₂) maintained at 37°C. After 10 minutes in the incubator, hepatocytes were dosed with 0.5 mL of pioglitazone at 20 μ M and placed back in the incubator. Final concentration of drug studied was 10 μ M in a total incubation volume of 1 mL (prepared to maintain <0.1% final DMSO concentration). At 1, 2 and 4 hours, the reaction was terminated by addition of acetonitrile and ethanol (80:20, 2 mL) with 1% acetic acid. An initial timepoint (t_0) was prepared by adding diluted hepatocytes and drug to a well already containing the organic solvent mixture. Precipitated protein was removed by centrifugation and the resultant supernatant was prepared for subsequent LC/MS analysis. Troglitazone was used as a positive GSH adduct control for cell viability.

LC/MS with neutral loss scanning. Samples from microsomal and hepatocyte incubations (1 mL) were prepared for analysis using a SPE 96-well plate (10 mg Oasis MCX, Waters Corp.) pre-conditioned with 1 mL of methanol and 1 mL of 2% TFA. The wells were washed with 0.1N hydrochloric acid (1 mL) followed by methanol (0.3 mL) and samples were eluted with 5% ammonium hydroxide in methanol (0.3 mL). Sample separation was achieved using a Hewlett-Packard 1100 HPLC system with gradient capabilities. A Waters Atlantis column (dC18 3 μ 2.1 x 50mm) was used with a sample injection volume of 50 μ L. The mobile phase system consisted of 0.1%

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formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B). The gradient conditions were 5% B (0-1 min), 5-95% B (1-3 min), 95% B (3-5.5 min), then back to initial conditions at 5.6 min. Samples were analyzed using an Applied Biosystems MDS Sciex API4000 in positive turboionspray mode. The detection method was a generic neutral loss method of 129 amu, which will detect any ion that has a neutral loss of 129 in a predetermined mass range (Chen, Zhang et al. 2001). Parameters for the mass spectrometer were set as follows: curtain gas 10 psi, temperature 600°C, declustering potential 71 volts, collision energy 21 volts. Raw data analysis was performed using Analyst v1.3.1 (Applied Biosystems, Toronto, Ontario Canada).

LC/MS with accurate mass measurement. Microsomal samples for analysis were evaporated to dryness and reconstituted in acetonitrile:0.1% formic acid (50:50). Sample separation was achieved using a Hewlett-Packard 1100 HPLC system with gradient capabilities. A Varian Polaris column (C18-A 3 μ 2.0 x 150mm) was used with a sample injection volume of 50 μ L. The mobile phase system consisted of 5 mM ammonium formate (pH 3.0) in water with 1% acetonitrile (solvent A) and acetonitrile (solvent B). The gradient conditions were 5% B, initial for 5 min, 5-100% B in 16 min, 100% B for 1.0 min, 100-5% B in 0.1 min, then hold at 5% B for 2.9 min. Samples were analyzed using a Micromass quadrupole time of flight (Q-ToF2) equipped with lockspray in positive ion electrospray mode. The instrument was operated in V-mode with approximately 10,000 resolution. Metergoline (Sigma) was used as a lockmass compound for MS and MS/MS accurate mass measurements. The instrument parameters were set as follows: Cone 50 volts, Capillary 3.25 kVolts, Collision 10 volts (27.5 for MS/MS), Source temp 100°C, Desolvation temp 250°C. Raw data analysis was performed using MassLynx 3.5 (Waters Corporation, Milford, MA).

Cleland's Reagent (dithiothreitol, DTT). DTT (100 mM) was added to an incubated and cleaned up HLM sample (100 μ L) containing pioglitazone and excess GSH (100 μ L, final concentration of DTT was 50 mM) and set at room temperature overnight. Sample analysis and separation was accomplished as stated above for accurate mass measurements. A HLM sample containing pioglitazone and excess GSH without DTT set at room temperature overnight as a control.

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Results

A summary of the novel metabolites, M-A and M-B, detected from incubations with rat and human liver microsomes and freshly isolated rat hepatocytes are shown in table 1. No glutathione conjugates were detected in freshly isolated (n=1) nor cryopreserved (n=2) human hepatocytes. In order to characterize the metabolism of pioglitazone we used two *in vitro* systems (microsomes and hepatocytes) and two analysis methods (LC/MS with neutral loss scanning and LC/MS Q-ToF accurate mass measurement). Glutathione conjugates, M-A and M-B, were first detected using LC/MS with neutral loss scanning and then further identified by LC/MS Q-ToF accurate mass measurements.

A review of the literature showed that pioglitazone is metabolized to form several oxidative products and a cleavage product at the ether linkage (Krieter, Colletti et al. 1994; Sohda, Ikeda et al. 1995; Tanis, Parker et al. 1996; Kiyota, Kondo et al. 1997; Eckland 2000; Shen, Reed et al. 2003). A majority of the oxidative metabolites previously reported for pioglitazone and conjugates M-A and M-B were detected in rat and human liver microsomes and hepatocytes in our lab (figures 2 and 3). Shen and colleagues (Shen, Reed et al. 2003) identified a thiazolidinedione ring-opened product (M-X) of pioglitazone in dog liver microsomes. This study reports the thiazolidinedione ring-opened product (M-X) identified in rat and human liver microsomes and two (M-A and M-B) novel thiazolidinedione ring-opened glutathione conjugates of pioglitazone detected in rat and human liver microsomes and freshly isolated rat hepatocytes.

Microsomal Studies

Both rat and human liver microsomes resulted in two peaks in the analysis corresponding to the typical 129 amu neutral loss of glutathione conjugates, which led to further identification by LC/MS Q-ToF accurate mass measurements.

Further analysis using rat and human liver microsomal incubation (5 mL) with pioglitazone, NADPH, and GSH was performed to increase sensitivity for detection of accurate mass for M-A, M-B, and M-X.

The extracted ion chromatogram of M-A and the product ion spectrum are shown in Figure 4A and 4B, respectively. M-A presented a $[M+H]^+$ ion of 636.2139 by LC/MS accurate mass measurement which corresponded to a molecular formula of $C_{28}H_{38}N_5O_8S_2$ (3.5 ppm). The product ion spectrum provided a typical neutral loss fragmentation of glutathione conjugates, m/z 561 (-75 amu) and m/z 507 (-129 amu). The accurate mass fragmentation outlined in Figure 5 supports the proposed structure of M-A.

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The extracted ion chromatogram of M-B and the product ion spectrum are shown in Figure 6A and 6B, respectively. M-B presented a $[M+H]^+$ ion of m/z 637.1979 by LC/MS accurate mass measurement which corresponded to a molecular formula of $C_{28}H_{37}N_4O_9S_2$ (3.5 ppm). The product ion spectrum provided a typical neutral loss fragmentation of glutathione conjugates, m/z 562 (-75 amu) and m/z 508 (-129 amu). The accurate mass fragmentation outlined in Figure 7 supports the proposed structure of M-B.

The extracted ion chromatogram of M-X and the product ion spectrum are shown in Figure 8A and 8B, respectively. M-X presented a $[M+H]^+$ ion of m/z 379 by LC/MS accurate mass measurement. The product ion spectrum gives a typical pattern showing a thiazolidinedione ring-opened product m/z 299.1757 $[M+H-SO_3]^+$, which corresponded to a molecular formula of $C_{18}H_{23}N_2O_2$ (0.9 ppm), and m/z 282.1448 $[M+H-SO_3-NH_3]^+$, which corresponded to a molecular formula of $C_{18}H_{20}NO_2$ (16.4 ppm). The product ion m/z 134.0912 suggests that the pyridyl ethyl end of the molecule was un-conjugated. This is the first detection of this metabolite of pioglitazone in rat and human *in vitro* systems.

Further positive identification of M-A and M-B was performed using Cleland's Reagent, DTT, which is known to reduce disulfide bonds (Cleland, 1964). The effect of using DTT is shown in figure 9, where the chromatographic peaks for M-A and M-B are present in the control (9A) and are approximately 96% reduced in the extracted ion chromatogram from an incubation of pioglitazone in HLM with excess GSH and DTT (9B).

Hepatocyte Studies

Hepatocyte extracts were also analyzed by LC/MS with neutral loss scanning, multiple reaction monitoring (MRM), and accurate mass measurements. M-A and M-B were detected in freshly isolated rat hepatocytes by neutral loss scanning and confirmed by MRM, comparison to control, and retention times of M-A and M-B isolated from rat and human liver microsomes. Glutathione conjugates, M-A and M-B, were not detected in freshly isolated nor cryopreserved human hepatocytes; however, several of the known oxidative metabolites of pioglitazone were detected (figure 2). The metabolites detected were M-IX, the -2H metabolite, M-III, the keto metabolite, M-II, M-IV, M-VII, M-VIII, the hydroxy metabolites, and M-V, the carboxylic acid metabolite on the terminal carbon of the ethyl side chain. Metabolites labeled in figures 2 and 3 were labeled using the identifiers from Shen (Shen, Reed et al. 2003).

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Discussion

Three thiazolidinedione ring-opened metabolites of pioglitazone have been identified by mass spectrometry in rat and human liver microsomes and in freshly isolated rat hepatocytes, two of which are glutathione conjugates. The ring-opened products were not positively identified in freshly isolated human hepatocytes nor cryopreserved human hepatocytes, even though the cells were not deficient in GSH activity as determined by a positive control, troglitazone. In each hepatocyte study, troglitazone was used as a positive control monitoring the MRM transition 747.0/440.0 (loss of GSH). This metabolite of troglitazone has previously been identified in cryopreserved human hepatocytes by Prabhu (Prabhu, 2002). The glutathione conjugate of troglitazone was identified in each hepatocyte (data not shown). This finding suggests that the *in vitro* glutathione conjugates of pioglitazone are specific to rat liver microsomes and hepatocytes, dog liver microsomes (Shen, Reed et al. 2003) and human liver microsomes. It is possible that these ring-opened products of pioglitazone are formed *in vivo* in human liver, but are not of significant consequence because either they are not relevant to toxicity or there are not significant amounts of the reactive species to cause toxicity.

The identified metabolically activated products of pioglitazone may have some clinical relevance for possible causes of liver injury; however, more extensive work would have to be performed to determine this. Therefore, the toxicological relevance of this finding is unclear, mainly because pioglitazone is a much safer drug from a human liver toxicity perspective compared to troglitazone. The safety profile of pioglitazone is much improved over troglitazone, which might be a result of dosage since pioglitazone is administered at approximately 10 times lower dosage compared to troglitazone.

This is the first report of a metabolically activated thiazolidinedione ring-opened product of pioglitazone in rat and human *in vitro* systems. The proposed structures of metabolites detected in this study are reported in Figure 10. Metabolites M-A, M-B, and M-X go through an oxidation step and then loss of carbon dioxide. Then the remaining amide-sulfenic acid intermediate can be further oxidized to M-X or go through nucleophilic substitution by GSH and lose a molecule of water to get M-A as a product.

Evidence exists in the literature that thiazolidinedione rings are known to be reactive species upon S-oxidation (Kassahun, Pearson et al. 2001; Nelson 2001) and pioglitazone follows in line in generating a ring-opened reactive species *in vitro*. As discussed earlier, troglitazone has been found to be hepatotoxic and removed from the market. There have been rare reports of clinically significant hepatocellular dysfunction and cholestatic liver injury in

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patients undergoing pioglitazone treatment (Maeda 2001; Chase and Yarze 2002; May, Lefkowitz et al. 2002). It is possible that these metabolites may contribute to these very rare reports of liver injury in an idiosyncratic fashion.

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Footnotes.

- a) None
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- c) None

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Legends for Figures

Figure 1. Structures of pioglitazone, troglitazone, and rosiglitazone

Figure 2. Metabolite profile of Pioglitazone after 4 hour incubation in freshly isolated human hepatocytes

Metabolites were labeled using the identifiers from Shen, Reed et al. 2003. Metabolites were identified by LC/MS accurate mass measurements and mass spectral fragmentation (data not shown)

Figure 3. Metabolite profile of Pioglitazone after 30 minute incubation in human microsomes with excess GSH

Phase 1 Metabolites were labeled as per figure 2. M-A and M-B are glutathione conjugates identified by LC/MS accurate mass measurements and mass spectral fragmentation

Figure 4. A) Extracted ion chromatogram (m/z 636) and B) product ion mass spectrum of M-A (11.38min), from an incubation in human liver microsomes with excess GSH

Figure 5. Proposed fragmentation of M-A based on accurate mass measurements

Figure 6. A) Extracted ion chromatogram (m/z 637) and B) product ion mass spectrum of M-B (11.8min), from an incubation in human liver microsomes with excess GSH

The peak at 11.38 min is the carbon¹³ peak of M-A.

Figure 7. Proposed fragmentation of M-B based on accurate mass measurements

Figure 8. A) Extracted ion chromatogram (m/z 379) and B) product ion mass spectrum of M-X (10.5 min), from an incubation in human liver microsomes with excess GSH

Figure 9. Extracted ion chromatograms of A) glutathione conjugates M-A and M-B from an incubation in human liver microsomes with excess GSH and B) M-A and M-B after an incubation with Cleland's Reagent, dithiothreitol

Figure 10. Proposed metabolic activation scheme for pioglitazone in rat and human liver microsomes and freshly isolated rat hepatocytes

* M-X previously identified in dog liver microsomes by Shen, Reed et al. 2003 and identified in this publication in rat and human liver microsomes. Steps described are not necessarily in the particular order of occurrence.

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Table I. Detection of novel pioglitazone metabolites in incubations with liver microsomes and freshly isolated hepatocytes.

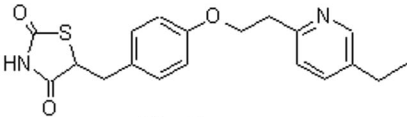
	Liver Microsomes		Freshly Isolated Hepatocytes	
	(Relative Abundance)			
Metabolite	Rat	Human	Rat	Human
M-A	+ (1.7%)	+ (2.5%)	+ (0.5%)	ND
M-B	+ (1.8%)	+ (0.2%)	+ (0.3%)	ND

+, metabolite detected

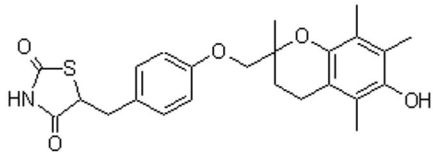
ND, not detected

%, the relative abundance of the metabolite peak area compared to the peak area of parent

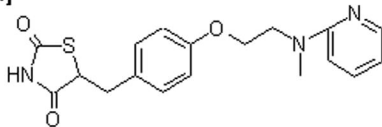
Figure 1



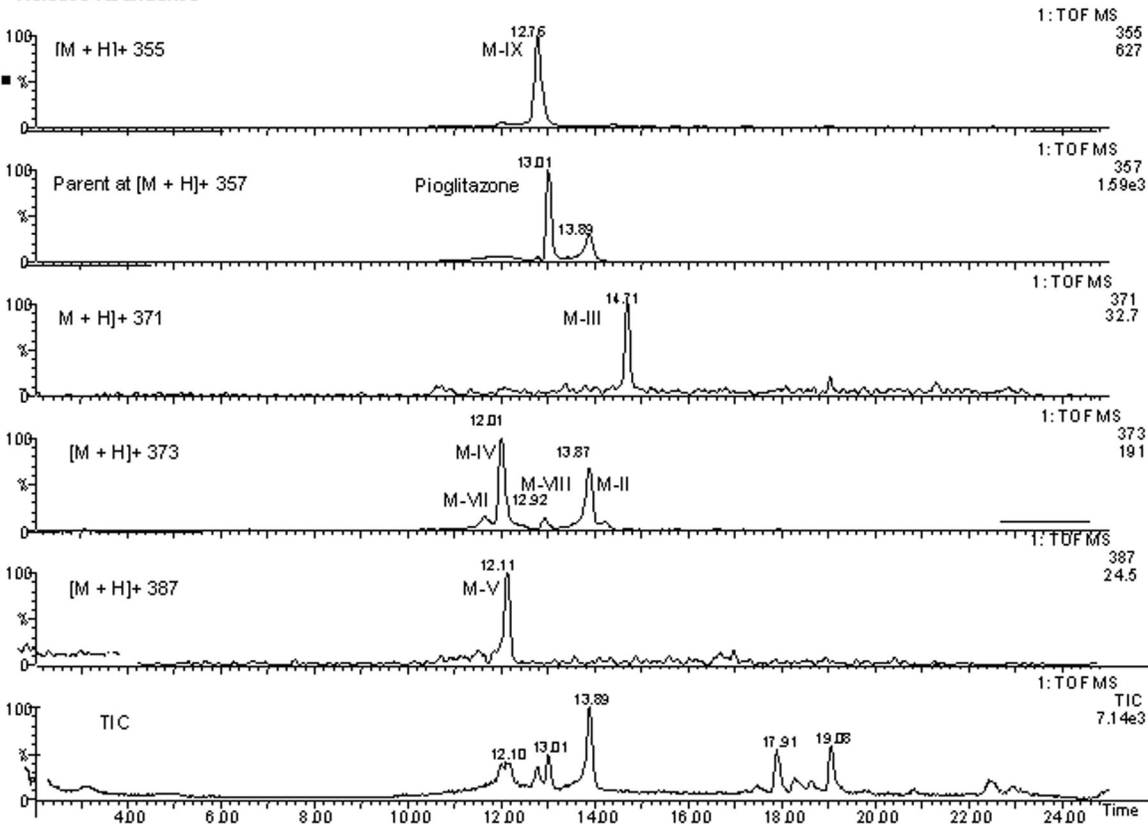
Pioglitazone
 m/z 357 $[M+H]^+$



Troglitazone



Rosiglitazone



Pio Human Mscb

Figure 3

Pio Human Mscb Sn (SG, 3/3)



Pio Human Mscb Sn (SG, 3/3)



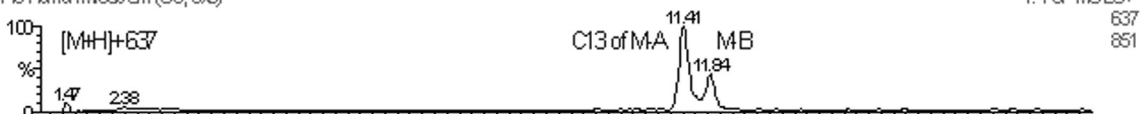
Pio Human Mscb Sn (SG, 3/3)



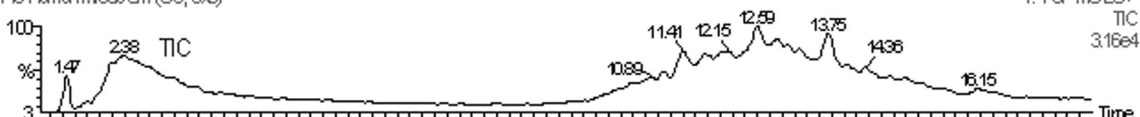
Pio Human Mscb Sn (SG, 3/3)



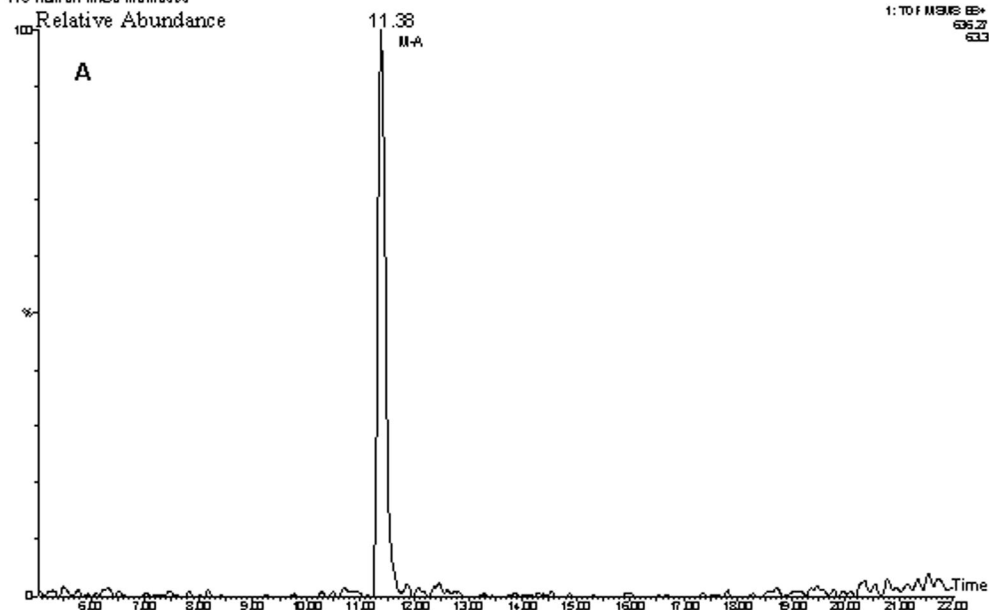
Pio Human Mscb Sn (SG, 3/3)



Pio Human Mscb Sn (SG, 3/3)



Plc Human MICS MSMS636

1: TO F MSMS 636
636.2
633

Plc Human MICS MSMS636

Relative Abundance

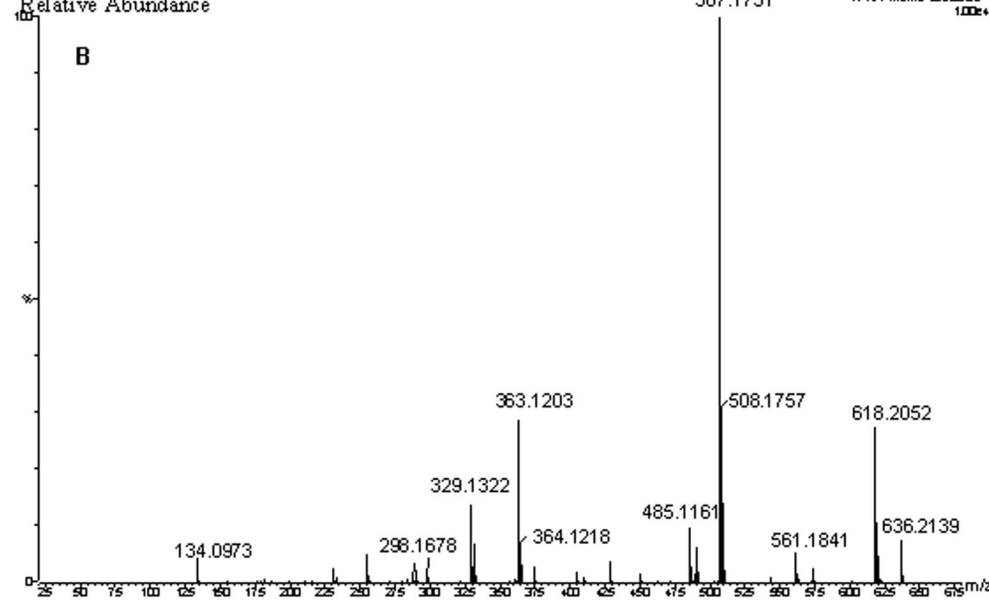
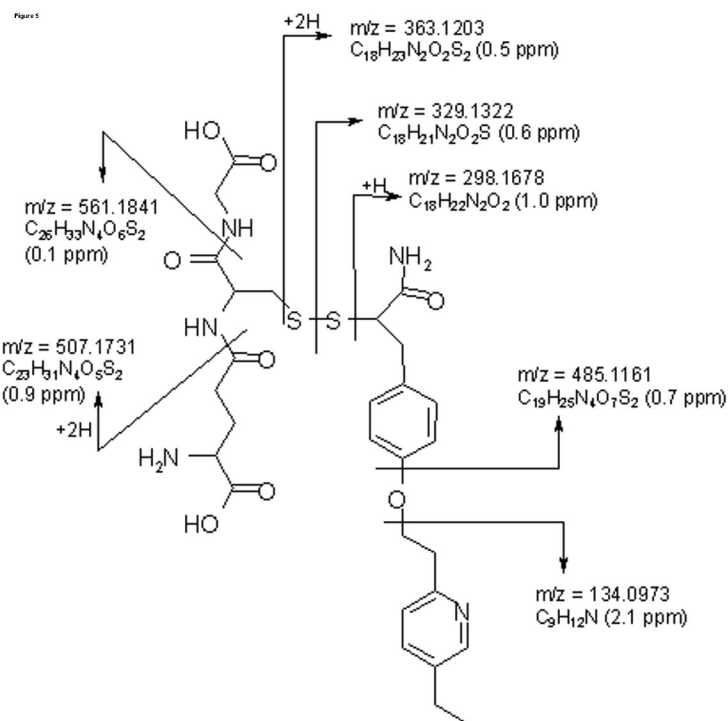
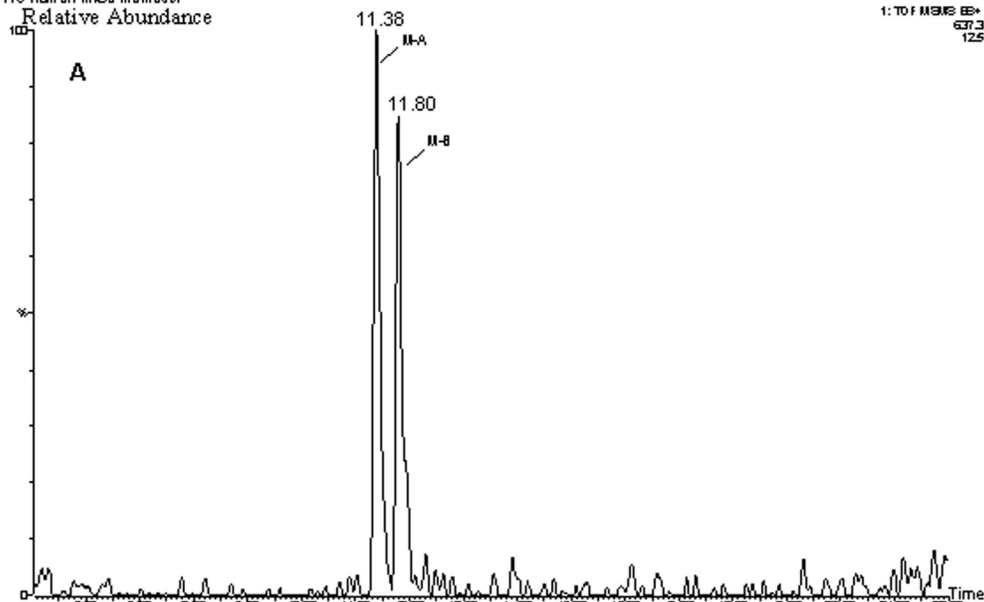
1: TO F MSMS 636.006
100%

Figure 5





Pro Human MICS MSMS637

Relative Abundance

1: TO F MSMS 637.0065
 1.7e3

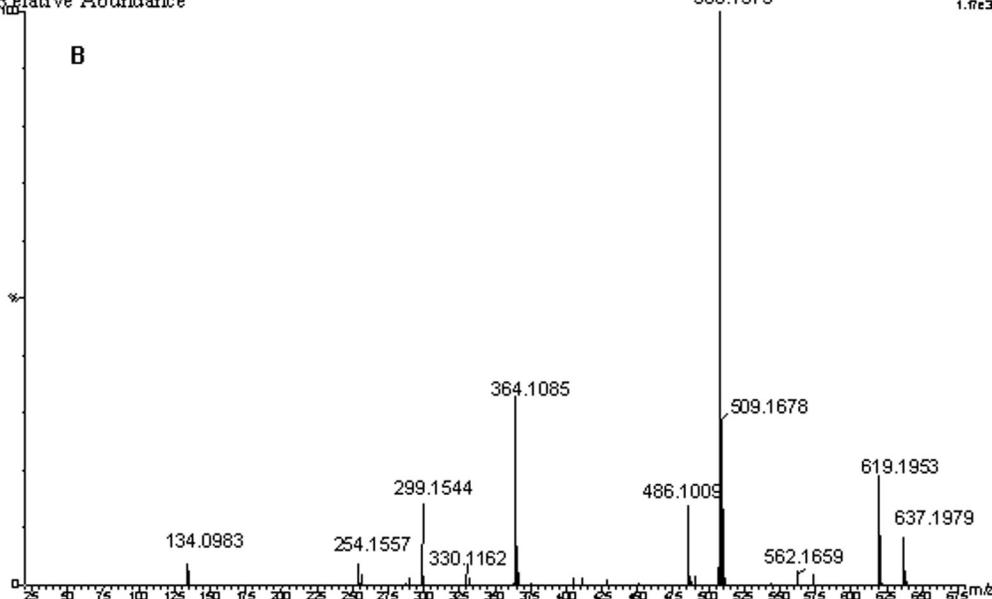
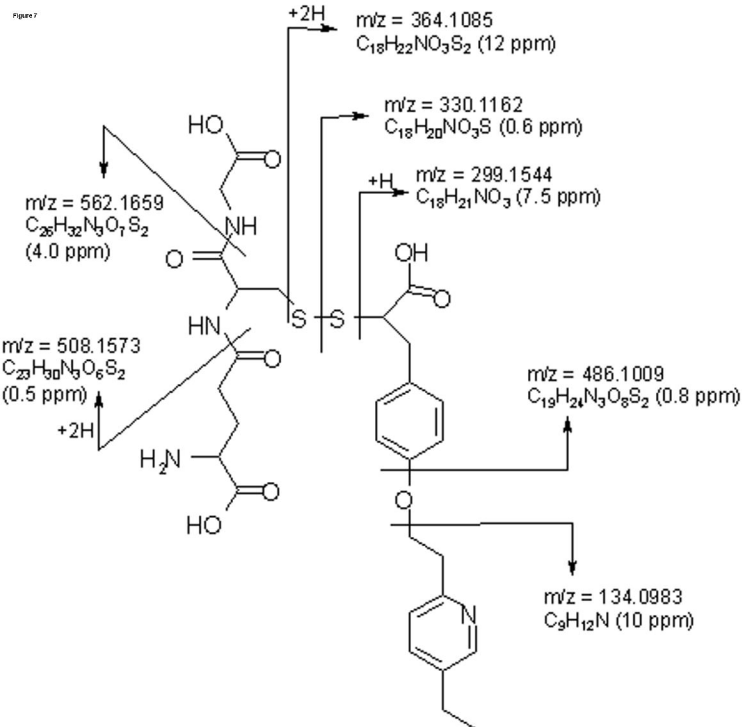
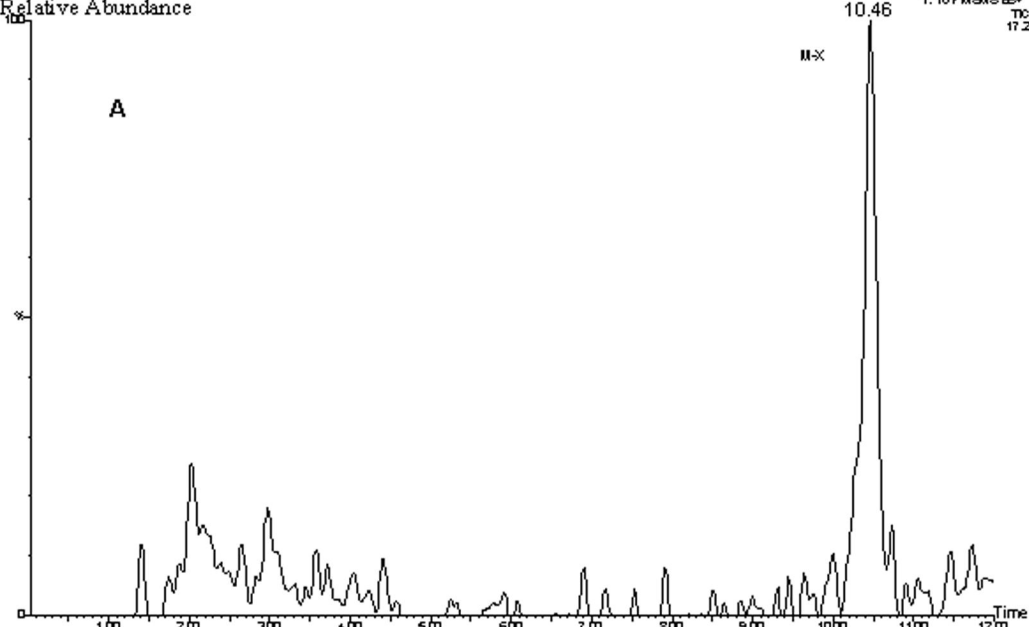


Figure 7

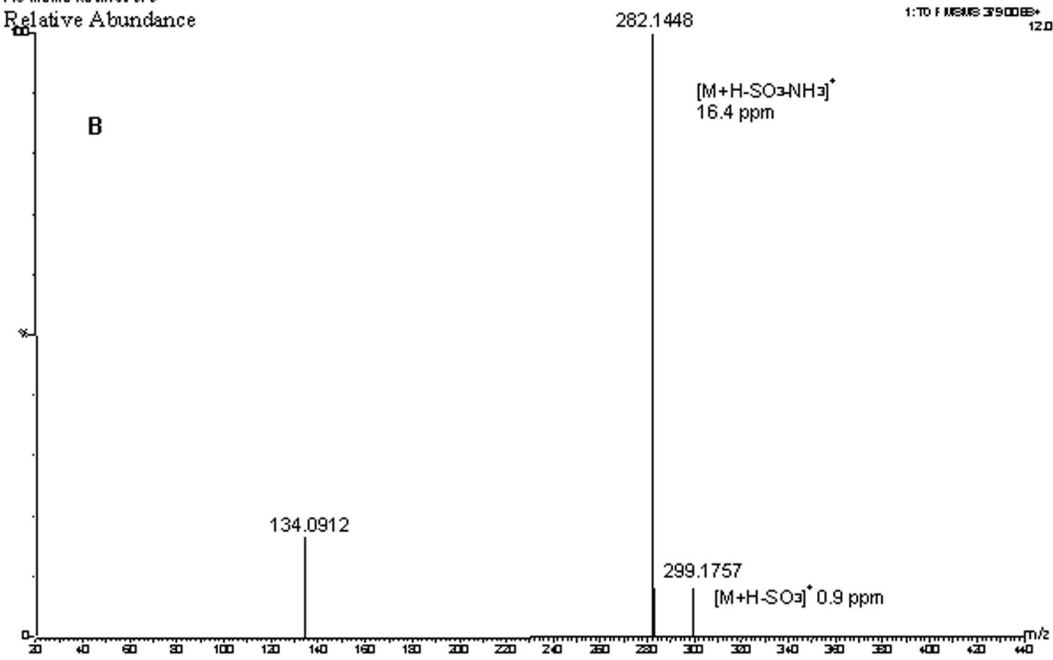


Relative Abundance

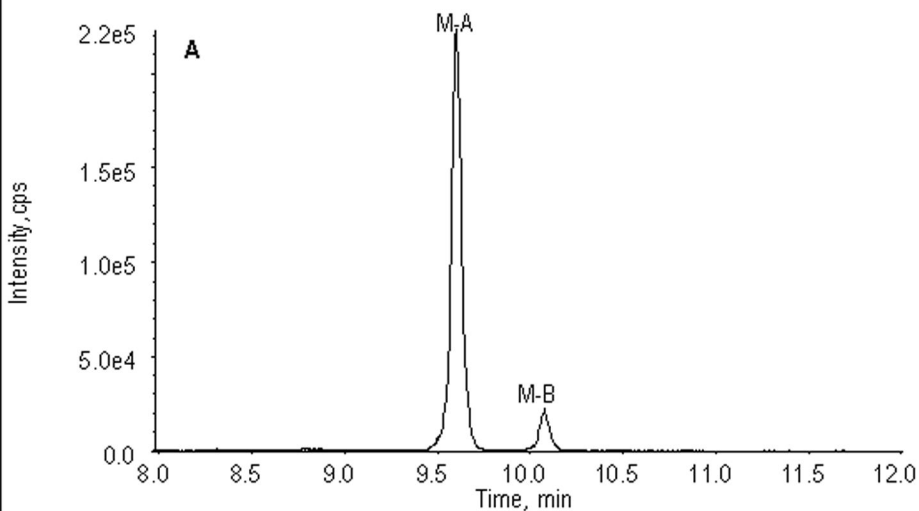


Plo MSMS RetTime 379

Relative Abundance



XIC of +MRM (2 pairs): 636.0/507.0 & 637.0/508.0 amu



XIC of +MRM (2 pairs): 636.0/507.0 & 637.0/508.0 amu

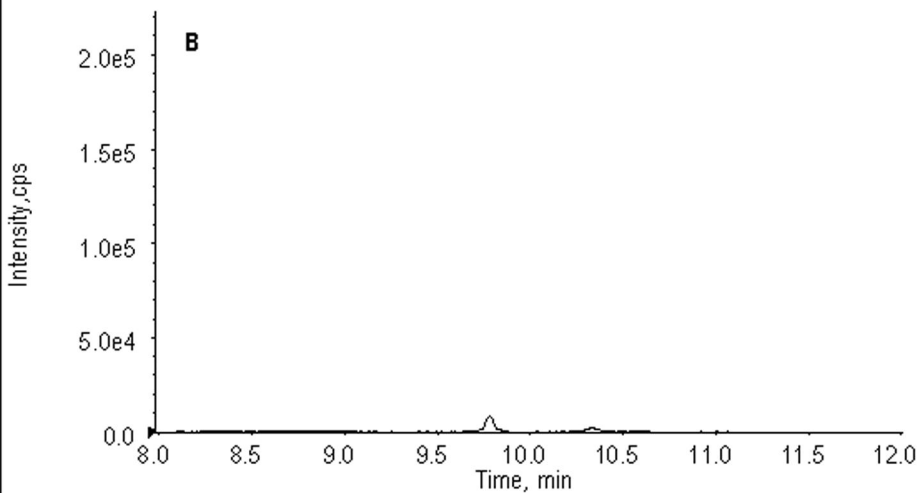


Figure 10

