Research Article***In vitro* metabolite identification of Tamsulosin using LC-Q-TOF mass spectrometry****Sai Laxmi Kesana^{1*}, Doble Jaya Prakash¹, Vamaraju Harinadha Babu², P.C .Sastry³**¹College of Technology, Osmania University- 500007, Telangana, India²G. Pulla Reddy College of Pharmacy, Mehdiapatnam, Hyderabad – 500028, Telangana, India³Institute of Engineers, Kolkata-700020, West Bengal, India

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Abstract

Background: Metabolite profiling has become one of the main drives in the drug discovery process to optimize pharmacokinetic properties and to increase the success rate of drugs. *In vitro* metabolism of Tamsulosin, the only alpha-1 adrenoceptor antagonist used in treatment of benign prostatic hyperplasia was investigated in several species. **Objective:** The main objective of this work is to build an *in vitro* methodology for identification of the probable metabolites and the extent of metabolism in various species. **Material and methods:** Tamsulosin was incubated with liver microsomes of Human, Rat and Dog. Simultaneous extraction and separation technique in combination with high resolution accurate mass spectrometric detector was used for the parent and the metabolite identification. The metabolites identified were confirmed by MS/MS and Accurate mass analysis using the analytical tool LC-Q-TOF Mass spectrometry. **Results and conclusion:** The major metabolic pathway was through oxidation and metabolites identified are M1, M2, M3, M4, M5 and M6. These are confirmed by MS/MS and Accurate mass analysis using the analytical tool LC-Q-TOF Mass spectrometry. Tamsulosin is more stable in human and less in rat showing there is species difference in its *in vitro* metabolism.

Keywords: Tamsulosin, Microsomes, UPLC, Xevo QTOF

Introduction

Tamsulosin is the only alpha-1 adrenoceptor antagonist used clinically in benign prostatic hyperplasia. The *in vivo* drug effect was due to the selective affinity of Tamsulosin and its metabolites towards the alpha-1D adrenoceptors, the selectivity and affinity of five metabolites were studied in rat and dog species (Soeishit et al., 2012), while the involvement of Cytochrome P450 isozymes in metabolism of Tamsulosin was investigated in by using 14C-tamsulosin in human (Taguchi et al., 1997). Drugs entering the body undergo biotransformation via Phase I and Phase II metabolic pathways, metabolites formed from Phase I reactions may likely lead to inactivation of drug, formation of active metabolite, and activation of inactive drug or formation of toxic metabolite (biotoxification) (Lemke et al., 2008).

In Tamsulosin the Phase I biotransformation resulted in the

formation of active metabolites which were well investigated *in vivo*, being metabolite identification has become important as part of the MIST and ICH Guidelines on drug safety. For the chemically reactive and pharmacologically active metabolites formed at greater than 10 percent of parent drug systemic exposure at steady state there is a definite need to characterize and evaluate nonclinical safety similar to the parent in drug safety assessment.

Recent efforts to improve the quality of later trials and to ensure that the safety of metabolites is adequately tested, the United States Food and Drug Administration have prompted the pharmaceutical industry to develop alternative strategies for assessing human exposure to metabolites earlier in the development process (Mort Shire-Smith et al., 2005, 2009; Chowdhury et al., 2006; Zhang et al., 2008). The success of this approach depends on the comprehensive detection and identification of relevant metabolites using *in vitro* sample (Lee and Liu 2007; Shu et al., 2008; Kalgutkar et al., 2005; Baillie, 2008).

The most challenging task in metabolite identification by LC/MS is the detection and structural elucidation of trace

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levels of unexpected metabolites in the presence of large amounts of complex interference ions from endogenous components (Zhang et al., 2005). Performing multiple sample extractions, concentrating sample extracts, and using several separation and detection methods are common strategies used to identify the metabolites. So the collection of data with regard to the formation of probable metabolites in various species is understood clearly using the *in vitro* models prior to *in vivo* screening (Wen and Fitch, 2009; Zhu et al., 2011).

Materials and methods

Chemicals

Tamsulosin purchased from Sigma Aldrich, Methanol and Acetonitrile from J.T Bakers, Potassium Phosphate Mono Basic (PBS) from Sigma Aldrich, Potassium Hydroxide (KOH) and Magnesium Chloride (MgCl₂) from Merck, NADPH Regenerating Solution (NRS) and Glucose - 6- Phosphate Dehydrogenase obtained from Sigma Aldrich, Formic acid from Fluka and the Microsomes were procured from Xenotech.

All the chemicals used are of analytical grade.

Procedure for *in vitro* sample preparation and analysis

Metabolite identification for the drug Tamsulosin was conducted in Human (HLM), Rat (RLM) and Dog liver microsomes (DLM) as metabolic profiles can vary across species both quantitatively and qualitatively, this testing paradigm usually is sufficient when the metabolic profile in humans is similar to that in at least one of the animal species used in nonclinical studies.

Oxidative microsomal incubations

Tamsulosin 10 μM was incubated with the liver microsomes (1.0 mg/ml in 100 mM phosphate buffer, pH 7.4) from human, rat and dog at 37°C for 60 minutes with 1 mM NADPH in a total volume of 0.5 ml.

At T60 mins incubations were stopped by adding 300 μL of ice-cold Acetonitrile containing 0.1% formic acid followed by centrifugation at 14,000 rpm for 10 minutes. The supernatants were concentrated and reconstituted using 50 μL of 90: 10 water and Acetonitrile with 0.1% Formic acid.

Mass Spectrometry analysis

High-resolution mass spectrometric (HR-MS) measurement was performed using Quadruple Time of Flight (QTOF) mass spectrometer (Waters) with dual orthogonal Z Spray ESI Source. Separation was performed by reverse-phase Ultra Performance Liquid Chromatography (Waters) using mobile phases (A _ 0.1% formic acid in H₂O; B _ 0.1% formic acid in Acetonitrile) for better resolution. The concentrated samples from the incubations were injected onto a 2.1250 mm, 5 μ, and C18 Polaris column at a flow rate of 0.4 ml/min at 25°C with a 60-min gradient (0–5min, 0% B; 5–50min, linear to 70% B, 51-55 min held to 90% B and thereafter 0% B till 60 min). The QTOF was operated under V-Mode and calibrated with polyethylene glycol; 50 pg/μL Leucine Enkephalin was used as lock spray at a flow rate of 3 μL/min, Electron Spray Ionization under positive ion mode and a collision energy of 25eV using scan MSe were used all along the retention window of 0 – 60 mins.

Results and discussion

The MS spectrum acquired after 60 minutes of incubation along the retention times for the resolved chromatographic peaks depicted the formation of various metabolites, the metabolite numbering was given based on their retention times starting from the retention time of Tamsulosin, the parent compound (Figure 2 and Table 1).

Figure 1. UPLC-MS/MS Chromatograph of RLM Blank used for Incubation

Figure 2. UPLC-MS/MS Chromatograph of Tamsulosin Following 60 minutes Incubation in RLM (Data used for structure identification of metabolites, Data collected by Xevo QToF)

Table 1. Metabolite Profiles of Tamsulosin in Human, Rat and Dog Liver Microsomes

M	m/z observed	Retention Time (min)	% of Total Metabolism			
			0 mins	60 mins		
				HLM	RLM	DLM
P	409.1490	27.893	100	47	15	32
M1	395.1286	25.903	ND	14	19	14
M2	425.1618	20.402	ND	17	10	16
M3	425.1488	19.367	ND	2	1	12
M4	381.1177	20.401	ND	14	20	21
M5	411.1498	19.677	ND	ND	7	3
M6	367.1169	18.051	ND	ND	15	2
Other Metabolites	NA	NA	ND	6	7	1

(Values in the table represent percent of parent and each metabolite at 0 and upon 60 minutes incubation) ND= not detectable (S/N < 3)

Detection of low levels of metabolites in complex biological samples would require the construction of theoretical probability based on the structure, which is done prior to data acquisition (Figure 3). Later the approach for drug metabolite identification with HR-MS, is accomplished via post-acquisition data mining where the chromatograph obtained were checked of their full-scan HR-MS and MS/MS data sets over the entire retention window.

Once metabolite ions were found, multistage product ion scans (MSⁿ) were carried out to obtain more detailed fragmentation pathways for structure elucidation. HR-MS instruments were

utilized for the determination of empirical formulae of metabolites and the MS/MS for their fragments. The mass-based identification was observed as the main annotation technique for, all the resolved chromatographic peaks.

This comprehensive approach was effective in identifying unexpected metabolites with multiple repetitions of several MS Scans.

In (Figure 1 and 2) full-scan HR-MS and MS/MS data sets were acquired with an intensity-dependent method and then processed. The process employed helped in finding expected metabolites by following predicted molecular

Figure 3. Proposed metabolic pathways for Tamsulosin (*Reference structures were drawn using the chembio software)

Table 2. Accurate Mass Analysis

M	Observed Mass	Theoretical mass	mDa	PPM	Formula
Parent	409.1490	409.519	-30.7	-17.5	$C_{20}H_{29}N_2O_5S$
M1	395.1286	395.492	-11.7	-19.6	$C_{18}H_{23}N_2O_6S$
M2	425.1618	425.518	-12.8	-13.1	$C_{20}H_{29}N_2O_6S$
M3	425.1488	425.475	10.6	14.9	$C_{19}H_{25}N_2O_7S$
M4	381.1177	381.465	-6.7	-17.6	$C_{17}H_{21}N_2O_6S$
M5	411.1498	411.492	14.4	15.0	$C_{18}H_{23}N_2O_7S$
M6	367.1169	367.439	8.4	12.9	$C_{16}H_{19}N_2O_6S$

Figure 4. MS/MS Spectra of Parent and Metabolites

masses based on the similarity of metabolites to those of the parent drug. The use of MS/MS of the parent drug and the metabolites proved to be effective in detecting the metabolites (Figure 4), while the elemental compositions and their respective accurate masses with a mass difference of less than 35 mDa

within a PPM error of less than 20 (Figure 5 and Table 2) gave a high assurance of the data generated thus aided in confirmation of the structures proposed. The co injection of analyte and the lock mass compound directly into ion source gave authenticated mass measurements all through the MS and MS/MS scans.

Figure 5. Elemental Composition of Parent and Metabolites

Conclusion

The untargeted, metabolic profiling of polar and non-polar metabolites with a comprehensive approach of single extraction

and using a single analytical platform was worked up using the drug Tamsulosin. The Tamsulosin metabolites were identified based on the theoretical interpretation in support to

the MS/MS and Elemental composition using Q TOF. Preliminary data after 60 min incubation in human, rat and dog suggested the formation of metabolites such as M1, M2, M3, M4, M5 and M6. The drug is rapidly metabolizing in rat liver microsomes in comparison to the human and dog liver microsomes. In human M2 and in rat and dog M4 were found to be major metabolites. However the drug is more stable in human compared to the rat and dog liver microsomes and the main metabolic pathway for Tamsulosin was observed through Oxidation.

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Conflicts of interest: Nil

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