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Study Protocol

Study Title: Development and Qualification of a Stereoselective Liquid Chromatography / Tandem Mass Spectrometry (LC/MS/MS) Assay for the Simultaneous Quantitation of Pentoxifylline and N-Acetylcysteine and Their Respective Major Metabolites in Human Plasma

BRI Study No: PAC-2008-001

Sponsor Study No: TBD

BRI Study Protocol No: Protocol-PAC-2008-001

Study Protocol Approval:

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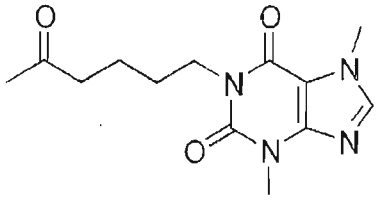
1. INTRODUCTION

PTL-202 is a fixed dose combination of two generic drugs consisted of pentoxifylline (1-(5-oxohexyl)-3, 7-dimethylxanthine), a methylxanthine derivative (Figure 1), and N-acetylcysteine (NAC), a strong antioxidant. The objective of this study is to develop an LC/MS/MS assay for the measurement of pentoxifylline and NAC along with their respective clinically relevant metabolites following oral administration of pentoxifylline alone, and following oral co-administration of pentoxifylline with N-



acetylcysteine (NAC), in human subjects in support of a Phase IIa clinical study.

Figure 1. Chemical Structure of Pentoxifyllin.

1.1 Clinical Pharmacokinetics of Pentoxifylline

After intravenous administration of pentoxifylline (200 mg), plasma levels declined in a biphasic manner, with a terminal $t_{1/2}$ of 1.63 +/- 0.8 hr. Plasma clearance was 1333 +/- 481 mL/min and the volume of distribution was 168 +/- 82.3 l [1].

Following single oral doses of 100, 200 and 400 mg of pentoxifylline as a solution, peak pentoxifylline plasma concentrations occurred between 0.29 and 0.41 hours. Cmax and AUC values increased in a dose-dependent manner for pentoxifylline over the three oral dose levels studied, while dose plasma AUC proportionality was also observed for the principal carboxylic acid metabolite [2]. The apparent plasma half-life of pentoxifylline varied between 0.39 and 0.84 hours over the oral doses studied [2]. The relatively shorter plasma half-life of pentoxifylline following oral administration compared with IV administration indicated a significant hepatic first-pass metabolism of pentoxifylline in humans.

In another study of pentoxifylline using ^{14}C-labelled drug in three healthy male subjects, peak plasma levels of radioactivity occurred 0.25-0.75 h after oral administration of 200 mg (50 µCi)[3]. Radioactivity decayed biexponentially with an initial plasma half-life of 1.01 +/- 0.13 h (distribution) followed by a terminal plasma half-life of 36.06 +/- 16.94 h. The peak plasma levels (0.48-2.25 µg/mL) of the parent drug, also occurred at 0.25-0.75 h and subsequently decayed extremely rapidly with an initial distribution plasma half-life of 0.18 +/- 0.15 h followed by a terminal plasma half-life of 0.76 +/- 0.44 h [3].

In a repeated oral dosing study of pentoxifylline using a sustained-release 400 mg dose three times daily for 9 days in 10 healthy subjects, accumulation of pentoxifylline in plasma was minimal [1]. The lack of pentoxifylline accumulation following repeated dosing is expected due to the relative short plasma half-life of the drug.

1.2 Metabolism of Pentoxifylline

Pentoxifylline is metabolized to at least seven metabolites in humans and animals, denotes as M1 to M7 [2 to 4], Figure 2. Reduction of the 5'-oxo group resulted in a major chiral hydroxy metabolite, M1 or 1-(5'-R,S-hydroxyhexyl)-3,7-dimethylxanthine. The R(-)-enantiomer of M1 or R(-)-M1 has been commonly referred as lisofylline. The S(+)-enantiomer of M1 or S(+)-M1 is however, a major enantiomeric component of M1, while lisofylline, or R(-)-M1, is a minor enantiomer of M1. The reduction of pentoxifylline to R(-)-M1 and S(+)-M1 can occur in the liver and in erythrocytes and this metabolic process is reversible.

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Therefore, during treatment of pentoxifylline, the plasma concentrations of pentoxifylline and the two enantiomeric M1 metabolites have been reported in equilibrium. Hepatic metabolism is also responsible for the formation of M3, M4 and M5 metabolites, while M5, a carboxypropyl derivative, is one of the two major metabolites of pentoxifylline.

Following oral and IV administration of pentoxifylline, the two major circulating metabolites (lisofylline or R(-)-M1 and M5) were at consistently higher plasma concentrations than the parent drug [1]. Lisofylline and the two homologous carboxylic acid metabolites of pentoxifylline showed plasma tmax values from 0.72 to 1.15 hours. The apparent plasma half-lives of the metabolites were in the range of 0.96 to 1.61 hours.

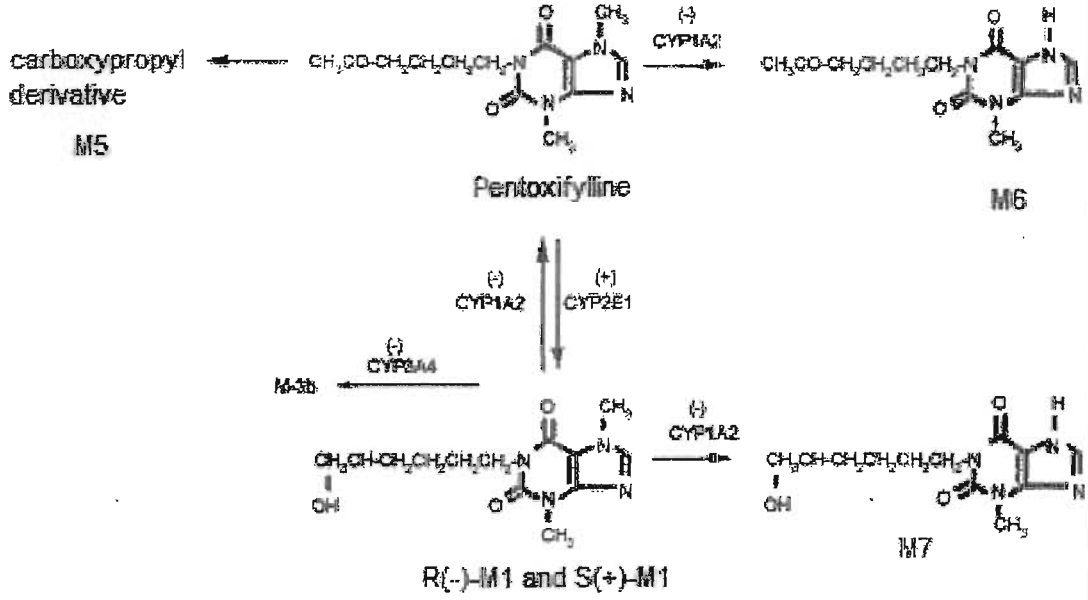

Figure 2. Chemical Structures of Pentoxifylline Metabolites.

From literature information, hepatic cytochrome P450 pathways including CYP3A4, CYP1A2 and CYP2E1 have been elucidated in the metabolism of pentoxifylline in human and animals [4-7]. Metabolism based drug-drug clinical interactions of pentoxifylline have been reported for ciprofloxacin [5, 6] and cimetidine [7].

In Cyp1A2 knockout mice, inhibition of CYP1A2 has resulted in markedly elevated levels in serum of pentoxifylline, suggesting CYP1A2 is involved in the metabolic clearance of pentoxifylline [5].

The metabolism of ^{14}C-labelled pentoxifylline after oral administration of 200 mg (50 microCi) to three healthy male volunteers indicated that pentoxifylline was rapidly absorbed and excreted with 89.1 +/- 2.4% of the ^{14}C material excreted in the urine over the first 6 hours. The dosed ^{14}C material (96.9 +/- 2.2%) was recovered in excreta by 24 h with 93.3 +/- 2.3% in the urine 3.0 +/- 0.2% in the faeces [3].

The metabolism of lisofylline and pentoxifylline were examined in the cytosol and microsomes prepared from four human livers to determine whether pentoxifylline is likely to serve as an efficient prodrug for the more active lisofylline, and to determine the extent to which lisofylline is converted back to pentoxifylline. Pentoxifylline is exclusively reduced to the optical antipode of lisofylline, *R*(-)-M1, in human liver cytosol,

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whereas the reduction in microsomes is 85% stereoselective in favor of S(+)-M1 formation. The less favored R(-)-enantiomer of M1 is accounting for 5-10% the total alcohol in blood.

The intrinsic clearance (Vmax/Km) of S(+)-M1 formation in cytosol was 4 times that in microsomes. In human liver microsomes, S(+)-M1 is exclusively converted to pentoxifylline, whereas approximately 45% of lisofylline oxidation is accounted for by the formation of pentoxifylline and the balance by aliphatic diols. It is concluded that pentoxifylline is an inefficient prodrug for delivery of lisofylline and that formation of pentoxifylline accounts for approximately 40% of the microsomal metabolites formed from lisofylline at substrate concentrations likely to be encountered in human therapeutic applications.

1.3 Clinical Pharmacokinetics of N-Acetylcysteine (NAC)

N-acetylcysteine (NAC) is a cysteine prodrug, which has been used clinically as a precursor of glutathione (GSH). The principal use of NAC pharmacologically is to replenish the cysteine and GSH stores in the body. Chemically NAC is similar to cysteine, however, NAC is less reactive than cysteine and their reaction with reactive oxygen species are several orders of magnitude lower than those of antioxidant enzymes such as superoxide dismutase (SOD), catalase and glutathione peroxidase. Therefore, the direct free radial scavenging activity of NAC is not likely to be of great importance for its antioxidant activity in vivo.

Pharmacokinetic studies of NAC have shown that NAC undergoes extensive first pass metabolism in the liver and kidneys to cysteine and cystine, resulting in very low plasma concentration of free NAC [21, 22], and virtually undetectable levels of NAC in other body fluids such as broncho-alveolar lavage [23]. Cysteine formed is then being utilised in the liver to produce GSH, which is released in circulation.

Plasma levels of cysteine, homocysteine and other low molecular-mass sulphydryls and disulfides metabolites in healthy adults at ages ranging from 21 to 92 have been previously studied [16]. Cysteine and homocysteine plasma levels were reported at 3 to 17 µM (363 to 2060 ng/mL) and 0.1 to 0.4 µM (13 to 54 ng/mL), respectively [16]. Changes in plasma cysteine and homocysteine levels are not related with age.

NAC, cysteine, and GSH are relatively unstable thiol containing compounds. For this reason, these compounds in plasma and are prone to be oxidized to their disulfide forms during sample collection and sample preparation, resulting in rapid decrease of the thiols and increase the levels of disulfides [11-12]. When GSH, cysteine or homocysteine was added to fresh human plasma, respectively 35%, 90%, and 88% decrease were observed by 5 min at room temperature [11]. Therefore, protecting the thiols from oxidation by converting them to stable derivatives is critical in each step from blood collection to plasma preparation and analysis. Reagents such as maleimides, Ellman's reagent, phenylisothiocynanate, ethacrynic acid, o-phthalaldehyde, monobromo-, and monochloro-bimane have been employed to the derivatization of active thiol containing compounds, such as GSH, cysteine and NAC [12-20]. These reagents have been shown to have different advantages and disadvantages.

To ensure the reliability of data collected for NAC, cysteine and cystine in the clinical study, there is a need of investigating and selecting a suitable chemical derivatizing reagent to be used for stabilising the thiols from sample collection to sample analysis. In this study, in vitro stability of NAC, cysteine and GSH will be investigated against the effect of the treatment of the container for blood collection, preparation of plasma (temperature and time of centrifugation), storage condition of plasma collected, means of protein precipitation, derivatizing condition (temperature, time, and pH), post-preparative storage conditions.

1.4 LC/MS/MS Stereoselective Assays of Pentoxifylline and its Major Metabolites

For quantitation of pentoxifylline and its active metabolite, lisofylline, a high-performance liquid chromatography tandem mass spectrometry (LC/MS/MS) assay has been published [8]. This LC/MS/MS assay relied on simultaneous multiple reaction monitoring of pentoxifylline (m/z 279/181) and lisofylline (m/z 263/181) along with 3-isobutyl-1-methylxanthine as an internal standard. Dichloromethane was used

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for extraction of pentoxifylline and the metabolite from plasma and a reversed-phase C8(2) 150 x 1.0 mm HPLC column was used to resolve all components within a 6 min assay. Calibration standards were established to be linear from 1 to 1000 ng/mL ($R^2 > 0.99$). This LC/MS/MS assay provided improvement in assay specificity over previously published HPLC assays, however, the use of a conventional reversed-phase HPLC column did not provide stereoselective measurement of lisofylline as the R(-)-M1 enantiomer and therefore data reported were misleadingly confounded with the major S(+)-M1 metabolite.

An HPLC stereoselective assay has been reported to provide chromatographic separation of the R(-)-M1 and the S(+)-M1 metabolite of pentoxifylline [9], and the HPLC assay has been applied to the study of lisofylline, specifically the R(-)-M1 enantiomer, in a placebo-controlled study of retinal blood flow changes by pentoxifylline and its metabolites in humans [10].

Based on this available stereoselective HPLC assay to enable the selective and specific measurement of the chiral metabolites of pentoxifylline, an LC/MS/MS assay will be developed in this study to provide specific and selective simultaneous measurement of pentoxifylline and its chiral M1 and achiral M5 metabolites.

1.5 LC/MS/MS Assays of N-Acetylcysteine (NAC) and its Major Metabolites

An LC/MS method was developed for simultaneous detection and quantitation of GSH, glutathione disulfide (GSSG), cysteine, homocysteine, and homocystine in biological samples (rat brain, lung, liver, heart, kidneys, erythrocytes and plasma) [19]. Thiols were derivatized with a large excess of Ellman's reagent to ensure an instantaneous and complete derivatization. The LLOQ of this method was 200 ng/mL plasma or 200 ng/g tissue, which was more sensitive than HPLC with UV detection and comparable to more sensitive than HPLC with fluorescence detection or electrochemical detection.

Recently, an LC/MS/MS assay has been published for the determination of reduced and oxidized glutathione and main precursors, including cysteine, in mice liver [20]. This assay employed iodoacedic acid to derivatize the thiols, with an assay LLOQ of 100 ng/mL tissue homogenate, based on 50µL sample.

In this study, an independent LC/MS/MS assay will be developed for quantitation of NAC, cysteine, cystine and glutathione in human plasma based on the results of derivatizing reagent selected.

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2. STUDY OBJECTIVES

To develop and qualify the assay performance of an electrospray stereoselective liquid chromatography / tandem mass spectrometry (LC/MS/MS) assay for the simultaneous quantitation of pentoxifylline and its major metabolites (lisofylline or R(-)-M1), S(+)-M1 and M5 in human plasma in support of a Phase-IIa pharmacokinetic investigation.

An independent LC/MS assay will be developed and its assay performance qualified for the simultaneous quantitation of N-acetylcysteine and its major metabolites cysteine, cystine and glutathione in human plasma in support of the same Phase-IIa pharmacokinetic investigation.

Anticipated clinical studies will involve oral administration of pentoxifylline alone and following oral co-administration of pentoxifylline with N-acetylcysteine (NAC) in human subjects. Therefore, measurement

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of pentoxifylline and its metabolites, along with measurement of NAC and its metabolites are required in support of pharmacokinetic assessment of the two co-administered drugs.

The stereoselective LC/MS/MS assay for pentoxifylline and the LC/MS assay for NAC are required to meet the pre-established assay qualification parameters defined in Experimental Section 3.0 of this study protocol.

3. EXPERIMENTAL

This assay development study is designed to establish two independent assay methods:

1) A stereoselective LC/MS/MS assay for the simultaneous quantification of pentoxifylline, its chiral M1 metabolites and M5 metabolite in human plasma.

2) In addition, an independent LC/MS assay for the quantitation of NAC and its major metabolite cysteine, cystine and glutathione in human plasma including sample collection and handling procedures, as well as storage stability data will be established.

Experimental work will be carried in three components. The first study component will involve the implementation of the stereoselective assay as described in the literature [9] and adapt the use of LC/MS/MS approach to provide specific and selective assay of pentoxifylline, its chiral M1 metabolites and M5 metabolite.

The second study component will involve the implementation and investigation of the effect of thiol derivatizing/stabilizing reagents on the stability of NAC, cysteine, cystine and glutathione during plasma collection, storage, preparation towards the assay and post-preparation storage steps. This will be incorporated with the
LC/MS/MS assay development and qualification for NAC, cysteine, cystine and glutathione in human plasma.

Upon completion of the assay development experiments, the third study component will involve performing the assay to qualify the assay performance against pre-established assay performance acceptance criteria described in this study protocol.

3.1 Development and Qualification of : A) a Stereoselective LC/MS/MS Assay for Pentoxifylline and its Chiral M1 Metabolite and M5 Metabolite; B) An Independent LC/MS Assay for N-Acetylcysteine and its Major Cysteine, Cystine and Glutathione Metabolites in Human Plasma

A stereoselective LC/MS/MS assay will be developed for the simultaneous quantitation of the following analyte components, each with specific assay LLOQ and sample requirements in Table 1:

Table 1. LC/MS/MS Quantitation Range and Assay LLOQ for Each Analyte Component.

Pentoxifylline and Metabolite components	Tentative Assay Calibration Range & Assay LLOQ	Sample Volume
Pentoxifylline	1 to 1000 ng/mL, LLOQ at 1 ng/mL	0.5 mL plasma or less
M1 chiral R(-) and S(+)-metabolites	1 to 1000 ng/mL, LLOQ at 1 ng/mL	No additional volume
M5 metabolite	1 to 1000 ng/mL, LLOQ at 1 ng/mL	No additional volume

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Pentoxifylline and Metabolite components	Tentative Assay Calibration Range & Assay LLOQ	Sample Volume
N-acetylcysteine (NAC)	100 to 10000 ng/mL, LLOQ at 100 ng/mL (to be confirmed with blank plasma)	TBD (1 mL or less)
Cystine	100 to 10,000 ng/mL, LLOQ at 100 ng/mL (to be confirmed with blank plasma)	TBD (1 mL or less)
L-cysteine	100 to 10000 ng/mL, LLOQ at 100 ng/mL (to be confirmed with blank plasma)	TBD (1 mL or less)
Glutathione	100 to 10000 ng/mL, LLOQ at 100 ng/mL (to be confirmed with blank plasma)	TBD (1 mL or less)

Each of the analyte components will be ex-vivo spiked in pooled human plasma (K2 EDTA) in establishing calibration standards and QC samples.

Calibration standards of pentoxifylline , its chiral M1 metabolite, M5 metabolite will be prepared and assayed over 11 levels (plus blank control) in duplicate (n=2) at 0, 1, 2, 5, 10, 20, 50, 100, 200, 500, 800 and 1000 ng/mL, based on 0.5 mL sample. QC samples (n=5) in pooled plasma at 5, 100 and 800 ng/mL will be prepared and assayed.

Calibration standards of NAC, cysteine, cystine and glutathione will be prepared and assayed over 8 levels (plus blank control) in duplicate (n=2) at 0, 100, 200, 500, 1000, 2000, 5000, 8000, and 10000 ng/mL, based on 1 mL or lower volume of plasma sample. QC samples (n=5) in pooled plasma at 300, 3000 and 9000 ng/mL will be prepared and assayed.

For each of the analyte components required for this study, the assay qualification experiments are summarized in Table 2.

Table 2. Qualification Parameters of a Stereoselective LC/MS/MS Assay for the Simultaneous Quantitation of Pentoxifylline, its Chiral M1 Metabolite and M5 Metabolite in Human Plasma, as well as an LC/MS assay for N-Acetylcysteine and its Major Cysteine, Cystine and Glutathione Metabolites in Human Plasma.

Method Parameters	Experimental	Method Performance Acceptance Criteria
System suitability	Evaluate by a minimum of 3 reproducible injections of an extracted sample spiked at low QC concentration	CV of assay injection reproducibility within 10%.
Calibration Standards	At least 8-level ex-vivo spiked pooled plasma calibration standards (n=2), plus blank control over the calibration range specified	Back-interpolated concentrations within 15% deviation (20% deviation at 15 pg/mL) from linear regression line for 75% of standards, coefficient of determination, r^2, greater than 0.99

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Assay Precision	Determined by ex-vivo spiked pooled plasma QC samples (n=5) at each of low, mid, and high concentration levels	CV within 15% at each level for 3 of 5 QC samples
Assay Accuracy	Determined by ex-vivo spiked pooled plasma QC samples (n=5) at each of low, mid, and high concentration levels	Within 15% deviation from the expected value at each level for 3 of 5 QC samples
Assay LLOQ / LOD	Determined by ex-vivo spiked pooled plasma at or below 1 ng/mL, based on 500 µL sample (n=5)	Within 20% deviation and within 20% CV from the expected LLOQ value in 3 of 5 QC samples
Assay Specificity	Determined by pooled blank plasma against other known pentoxifylline metabolites in human, also include stereoselectivity of the assay against R(-)-M1 and S(+)-M1, NAC, cysteine and the internal standards to be used in both assays	Free of chromatographic and mass spectral assay interference
Assay Recovery/ Plasma Matrix Effect	Determined by ex-vivo spiked pooled plasma QC samples (n=5) at each of low, mid, and high concentration levels	Desirable absolute assay recovery exceeding 70% and lack of significant matrix effect

3.3 Validation of the Stereoselective LC/MS/MS Assay

Validation of the stereoselective LC/MS/MS assay developed will be based on the assay performance established during this method development study. The validation experiments are independently proposed and performed according to BRI Standard Operating Procedures (SOPs) and with reference to applicable GLP regulations.

4. REPORT

A draft study report on the conduct of this bioanalytical chemistry study will be available to the Sponsor within two weeks following completion of experimental work. Revisions to the initial draft report will be provided to the Sponsor as a final report released by the QAU of BRI two weeks following receipt of revision comments from the Sponsor.

The study report will include, but not limited to, the report components listed below:

- Authentication
- QAU Statement
- Table of Content
- Lists of Abbreviations, Figures, Tables and Appendices
- Report Summary
- Introduction
- Study Objectives
- Experimental Methods and Procedures
- Instrumentation and Equipment
- Data Management
- Results and Discussion
- Conclusion
- A copy of the approved study protocol, all protocol amendments, and all protocol deviations that may affect the integrity of the study will be included in the study report.

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5. STUDY SAMPLES AND MATERIALS

The following reference standards will be obtained from a qualified commercial supplier for this study.

Reference Standard Materials	Quantity
pentoxifylline	500 mg
M1, R(-)enantiomer	100 mg
M1, S(+)enantiomer	100 mg
M5 metabolite	500 mg
N-acetylcysteine (NAC)	500 mg
L-cysteine	500 mg
cystine	500 mg
glutathione	500 mg
An internal standard for pentoxifylline and its metabolites (to be determined)	To be determined
An internal standard for NAC (to be determined, e.g. cysteamine)	To be determined

Metabolites reference standards and internal standards are independently provided by qualified custom synthesis companies along with Certificate of Analysis.

All other study supplies and materials associated with this study will be provided by BRI. All materials used for the study will be documented in the Study Report.

6. LC/MS/MS EQUIPMENT

A Quattro®-Micro or Quattro®-LC LC/MS/MS system will be operated in the MS/MS mode with an Agilent Model 1100 liquid chromatograph equipped with an automated refrigerated autosampler. Instrument control, operating parameters and instrument data acquisition will be carried out using an automated software system according to data handling procedures established at BRI. A description of the sample preparation and analytical equipment used for this study will be noted in the Analytical Study Report.

7. GLP COMPLIANCE

The developmental nature of this experimental study does not require to be conducted in conformance with the following applicable Good Laboratory Practice (GLP) Regulations/Standards/Guidelines:

- United States Food and Drug Administration, Title 21 Code of Federal Regulations Part 58, current.
- Organization for Economic Cooperation and Development, The OECD Principles of Good Laboratory Practice, Series on Principles of Good Laboratory Practice and Compliance Monitoring, Monograph No.1 to 14, current.
- Japanese Ministry of Heath and Welfare, Ordinance No. 21, April 1, 1977.

The Study Director at BRI, in accordance with BRI Biopharmaceutical Research Inc. Standard Operating Procedures (SOPs), will review the study protocol and the final report.

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8. STUDY ARCHIVES

All experimental raw data, related documentation and the study report will be archived according to the BRI SOP-QA-008 version 5.1 for a minimum of 10 years, unless alternative arrangement is made by written request of the Study Sponsor.

END of STUDY PROTOCOL

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Study Proposal

Study Title:	**Development and Qualification of a Stereoselective Liquid Chromatography / Tandem Mass Spectrometry (LC/MS/MS) Assay for the Simultaneous Quantitation of Pentoxifylline and N-Acetylcysteine and Their Respective Major Metabolites in Human Plasma**
BRI Study No:	**PAC-2009-001**
Sponsor Study No:	**TBD**
BRI Study Protocol No:	**Protocol-PAC-2009-001**

Study Proposal Approval:

Clara Faan.
VP Business Development
BRI Biopharmaceutical Research Inc.

12/10/09
Date

Doug Unwin
President
Pacific Therapeutics Ltd.

Dec 10/09
Date

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Timelines and Cost Proposal

1. TIMELINES AND PROJECT MILESTONES

BRI is ready to perform this study in reference to Study Protocol: PAC-2009-001 approximately 10 working days upon the approval of this Study Protocol by the Sponsor, subject to BRI receiving all materials and supplies for this study.

2. ESTIMATED COSTING AND BUDGET

Study Components	Total Cost (CAN)
Development and Qualification of a Stereoselective LC/MS/MS Assay for Pentoxifylline and its Chiral M1 Metabolite and M5 Metabolite in Human Plasma	[redacted]
Development and Qualification of a LC/MS Assay for N-Acetylcysteine and its Major Cysteine, Cystine and Glutathione Metabolite in Human Plasma	[redacted]
Study Summary for each assay	inclusive

Reference Standard Materials	Quantity
pentoxifylline	[redacted] 100 mg
M1, R(-)enantiomer	[redacted] 100 mg
M1, S(+)enantiomer	[redacted] 100 mg
M5 metabolite 1-(3-Carboxypropyl)-3,7-dimethylxanthine	[redacted] 50 mg
N-acetyl-L-cysteine (NAC)	[redacted] 500 mg
L-cysteine	[redacted] 500 mg
L-cystine	[redacted] 500 mg
Reduced L-glutathione	[redacted] 500 mg
Deuterium stable-isotope labelled d6-pentoxifylline as an assay internal standard (to be determined)	Please see attachment for price quotation, [redacted] 50 mg, 4-6 weeks delivery
Deuterium stable-isotope labelled NAC as an internal standard for NAC (to be determined, e.g. cysteamine)	Please see attachment for price quotation, [redacted] mg (in stock)

3. PAYMENT TERMS

The estimated cost of this study in Canadian funds is payable at [redacted]% at the commencement of the study. The balance 40% is payable at completion of the study and [redacted] is payable at the acceptance of a final Study Report.

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4. GENERAL CONDITIONS

4.1 The prices quoted in this study proposal are in Canadian funds and subjected to BRI receiving all necessary reference standard test materials to conduct the study outlined.

4.2 The anticipated personnel required to perform the above study at BRI will include a Study Director. An analytical team will consist of a Study Director, Research Scientist and Instrumentation Lab Supervisor.

4.3 All experimental work will be carried out by BRI on a best-effort-basis in good faith of the scope of work described in this proposal in context with any exploratory and investigative nature of the study.

4.4 Any work or services requested by the Sponsor in addition to those covered in this original protocol will be described in an addendum (Testing Confirmation) and the costing submitted to the Sponsor separately for approval.

4.5 This Study Proposal constitutes a standalone Agreement and understanding between the parties with respect to the subject matter herein without any reference to prior Agreements or understandings. This Agreement may be modified or amended only by a written document signed by a designated representative from each of BRI and the Sponsor.

For acceptance of this Study Proposal in agreements with the terms and procedures described in the referenced Study Protocol, please provide authorised signatures as approval for BRI to proceed with the work described herein.

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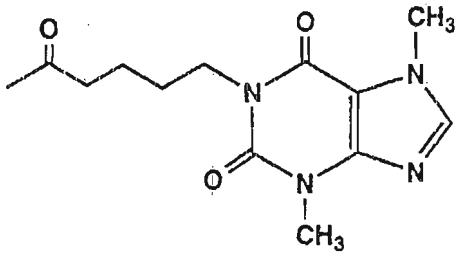
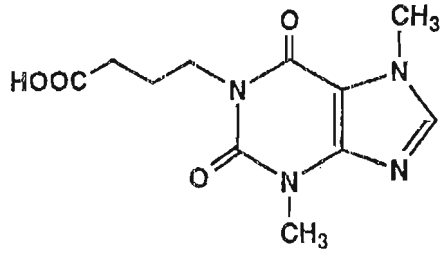
Attachment 1: Reference standards and metabolite standards and internal standards for study PAC-2008-001

Common Name (CAS)	Chemical Name	Comment	Amount	Budget (note)
Pentoxifylline, Trental® (Hoechst) (**CAS**-6493-05-6)	1-(5-oxohexyl)-3, 7-dimethylxanthine	Isolate and purify from Trental	[redacted]	[redacted]
D6-pentoxifylline	Deuterium on any of the two N-methyl	Custom synthesize	[redacted]	[redacted]
Pentoxifylline metabolite 5	1-(3-carboxypropyl)-3, 7-dimethylxanthine	Custom synthesize	[redacted]	[redacted]
Lisofylline	(±)-1-(5-Hydroxyhexyl)-3,7-dimethylxanthine	Biomol (attached)	[redacted]	[redacted]
N-acetyl-L-cysteine (616-91-1)		Sigma	[redacted]	[redacted]
D3-N-acetyl-L-cysteine	Deuterium on acetyl group	Custom synthesize	[redacted]	[redacted]
Reduced L-glutathione		Sigma	[redacted]	[redacted]
L-cysteine		Sigma	[redacted]	[redacted]
L-cystine		Sigma	[redacted]	[redacted]

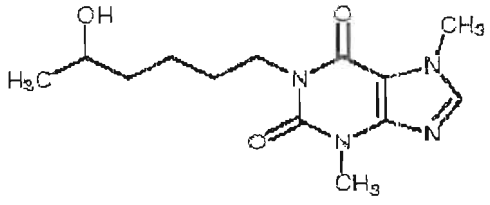


Pentoxifylline



Metabolite 5

Note: Prices subject to actual cost from suppliers.



Lisofylline
(±)-1-(5-Hydroxyhexyl)-3,7-dimethyl xanthine

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PRODUCT DATA

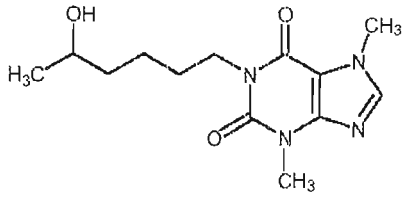
PRODUCT: Lisofylline
(±)-1-(5-Hydroxyhexyl)-3,7-dimethyl xanthine

CAS NO. 6493-06-7

CATALOG NO.: LP-102

LOT NO.: temp

STRUCTURE:



PHYSICAL APPEARANCE: White solid

MOLECULAR FORMULA: $C_{13}H_{20}N_4O_3$ MOLECULAR WEIGHT: 280.3

PURITY: 98% (TLC)

SOLUBILITY: Soluble in DMSO

STORAGE: Store, as supplied, at room temperature for up to 1 year.
Store solutions at -20°C for up to 3 months.

APPLICATION NOTES: Potent inhibitor of phosphatidic acid generation (IC_{50} = 0.6 μM).[1] Protects mice from endotoxic shock[1] and attenuates sepsis-induced acute lung injury in pig[2]. Novel anti-inflammatory agent, which enhances glucose-stimulated insulin secretion[3]. Inhibits IL-12-mediated STAT4 activation[4]. The product is not sterile.

REFERENCES:
1. G.C.Rice *et al. Proc.Natl.Acad.Sci.USA* 1994 **91** 3857
2. N.Hasegawa *et al. Am.J.Respir.Crit.Care Med.* 1997 **155** 928
3. J.S.Striffler & J.L.Nadler *Metabolism* 2004 **53** 290
4. Z. Yang *et al. Ann N.Y. Acad. Sci.* 2003 **1005** 409

The pharmacological and toxicological properties of this product have not been fully investigated. Exercise caution in use and handling. This product must not be used in humans.