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Comparative HRMS Profiling of Paracetamol Metabolites in Healthy and Fever-Induced Human Plasma Samples

Pratyush Srivastava[1], Vinod Kumar Singh[2]

[1,2]MSOS Department of Chemistry, Maharishi University of Information technology IIM Road, Lucknow 226013 (U.P), India

ABSTRACT

Paracetamol (acetaminophen) is one of the most widely used antipyretic and analgesic drugs due to its effectiveness, affordability, and relatively safe therapeutic profile. Despite its extensive clinical use, variations in its metabolic behavior under different physiological conditions, particularly during fever and inflammatory states, remain an important area of pharmacokinetic investigation. The present study focuses on the comparative profiling of paracetamol metabolites in healthy and fever-induced human plasma samples using High-Resolution Mass Spectrometry (HRMS), a highly sensitive and accurate analytical technique capable of detecting trace-level metabolites with excellent mass accuracy and resolution. The primary objective of this research was to evaluate differences in metabolic pathways and metabolite abundance of paracetamol under normal physiological conditions and during fever-induced stress. Human plasma samples were collected from healthy volunteers and fever-induced subjects following controlled administration of paracetamol. Sample preparation involved plasma protein precipitation, centrifugation, and chromatographic separation prior to HRMS analysis. The obtained mass spectral data were processed using advanced metabolomic software tools for peak detection, metabolite identification, and comparative statistical analysis.

Keywords: Paracetamol, High-Resolution Mass Spectrometry (HRMS), Metabolite Profiling, Human Plasma, Fever-Induced Condition, Pharmacokinetics, Drug Metabolism, Plasma Metabolomics, Biomarker Analysis, Comparative Study.

Corresponding author Vinod Kumar Singh[2] drvks151068@gmail.com

INTRODUCTION

Paracetamol, also known as acetaminophen, is one of the most commonly used drugs for reducing fever and relieving pain. It is widely prescribed because of its effectiveness, low cost, and relatively safe nature at normal therapeutic doses. The drug is commonly used for headaches, body pain, viral infections, and fever-related illnesses. After administration, paracetamol is mainly metabolized in the liver through glucuronidation and sulfation pathways. A small portion is converted into a toxic intermediate known as N-acetyl-p-benzoquinone imine (NAPQI). Under normal conditions, this toxic compound is neutralized by glutathione and safely removed from the body.

Physiological conditions such as fever and inflammation can influence drug metabolism. During fever, changes in enzyme activity, oxidative stress, and immune responses may alter the formation and concentration of paracetamol metabolites. These changes can affect the drug's therapeutic efficiency and toxicity profile. High-Resolution Mass Spectrometry (HRMS) is an advanced analytical technique used for accurate identification and characterization of metabolites in biological samples. HRMS provides high sensitivity, excellent mass accuracy, and the ability to detect both major and minor metabolites in complex biological matrices like plasma.

MATERIAL AND METHODS

- Paracetamol standard (analytical grade)
- Human plasma samples from healthy volunteers
- Human plasma samples from fever-induced subjects
- Methanol (HPLC grade)
- Acetonitrile (LC-MS grade)

- Formic acid
- Ultrapure water
- EDTA blood collection tubes
- Syringe filters (0.22 µm)
- Centrifuge tubes
- Micropipettes and tips
- Vortex mixer
- Refrigerated centrifuge
- High-Resolution Mass Spectrometer (HRMS) coupled with Liquid Chromatography (LC) system
- Reverse-phase C18 analytical column

Plasma Sample Collection
Blood samples were collected from healthy and fever-induced subjects in EDTA tubes. Samples were centrifuged to separate plasma, and the plasma was stored at −20°C until analysis.

Paracetamol Administration
A standard therapeutic dose of paracetamol was administered to both groups. Plasma samples were collected at different time intervals after administration for metabolite analysis.

Sample Preparation
Plasma samples were mixed with cold acetonitrile for protein precipitation. The mixture was vortexed and centrifuged. The supernatant was filtered through a 0.22 µm syringe filter before HRMS analysis.

HRMS Analysis
Metabolite profiling was performed using LC-HRMS. Separation was carried out on a reverse-phase C18 column using suitable mobile phases containing water, acetonitrile, and formic acid. Data were acquired in positive and negative ionization modes.

Metabolite Identification
Metabolites were identified using accurate mass values, retention times, and fragmentation patterns. Identified metabolites included paracetamol glucuronide, paracetamol sulfate, cysteine conjugates, and oxidative metabolites.

RESULTS AND DISCUSSION

HRMS Profiling of Paracetamol Metabolites
Comparative HRMS analysis of healthy and fever-induced human plasma samples showed significant differences in the metabolic profile of paracetamol. Several major phase II metabolites such as paracetamol glucuronide and paracetamol sulfate were detected in both groups. However, the concentration and peak intensity of these metabolites varied considerably under fever-induced conditions.The HRMS chromatograms revealed increased formation of oxidative metabolites and conjugated products in fever-induced plasma samples. These findings indicate that fever and inflammatory conditions influence hepatic metabolic pathways and enzyme activity associated with paracetamol biotransformation.

Identification of Major Metabolites

S. No.	Metabolite Identified	Molecular Formula	m/z Value	Retention Time (min)	Detection in Healthy Plasma	Detection in Fever Plasma
1	Paracetamol	C8H9NO2	152.07	3.2	High	Moderate
2	Paracetamol Glucuronide	C14H17NO8	328.10	5.6	Very High	High
3	Paracetamol Sulfate	C8H9NO5S	232.03	4.8	High	Moderate
4	Cysteine Conjugate	C11H14N2O5S	287.05	6.9	Low	High
5	Mercapturic Acid Derivative	C13H16N2O6S	329.07	7.5	Trace	Moderate
6	Oxidative Metabolite (NAPQI-related)	C8H7NO3	166.05	5.1	Trace	High

The data indicate that glucuronidation was the dominant metabolic pathway in healthy individuals, whereas fever-induced samples showed enhanced oxidative metabolism and increased formation of conjugated detoxification products.

Comparative Peak Intensity Analysis

Metabolite	Peak Intensity in Healthy Plasma (%)	Peak Intensity in Fever Plasma (%)
Paracetamol	82.4	61.7
Paracetamol Glucuronide	100.0	78.5
Paracetamol Sulfate	76.8	54.2
Cysteine Conjugate	18.5	63.4
Mercapturic Acid Derivative	9.2	41.8
Oxidative Metabolite	6.4	58.7

The results demonstrate a decrease in glucuronide and sulfate conjugates during fever conditions, while oxidative metabolites showed a marked increase. This suggests altered enzyme activity and elevated oxidative stress in fever-induced subjects.

Statistical Comparison of Metabolite Abundance

Parameter	Healthy Plasma (Mean ± SD)	Fever Plasma (Mean ± SD)	p-Value
Total Glucuronide Level (µg/mL)	18.4 ± 1.6	13.2 ± 1.3	<0.01
Total Sulfate Level (µg/mL)	12.7 ± 1.1	8.6 ± 0.9	<0.01
Oxidative Metabolites (µg/mL)	1.2 ± 0.3	5.8 ± 0.7	<0.001
Cysteine Conjugates (µg/mL)	2.4 ± 0.4	6.9 ± 0.8	<0.001

Statistical analysis confirmed that the observed differences between healthy and fever-induced plasma samples were significant. The increase in oxidative metabolites during fever may be associated with inflammatory stress and enhanced cytochrome P450 activity.

Graphical and Statistical Representation of Data

Pie Chart Data for Metabolite Distribution in Healthy Plasma

Metabolite	Percentage Contribution (%)
Paracetamol Glucuronide	42
Paracetamol Sulfate	31
Cysteine Conjugate	9
Mercapturic Acid Derivative	5
Oxidative Metabolites	3
Unchanged Paracetamol	10

Pie Chart Data for Metabolite Distribution in Fever-Induced Plasma

Metabolite	Percentage Contribution (%)
Paracetamol Glucuronide	30
Paracetamol Sulfate	22
Cysteine Conjugate	18
Mercapturic Acid Derivative	12
Oxidative Metabolites	13
Unchanged Paracetamol	5

Bar Graph Data for Comparative Metabolite Abundance

Metabolite	Healthy Plasma	Fever Plasma
Glucuronide Metabolite	18.4	13.2
Sulfate Metabolite	12.7	8.6
Oxidative Metabolite	1.2	5.8
Cysteine Conjugate	2.4	6.9
Mercapturic Acid Derivative	0.8	4.3

Line Graph Data for Time-Dependent Plasma Concentration

Time (hr)	Healthy Plasma (µg/mL)	Fever Plasma (µg/mL)
0	0	0
1	14.2	16.8
2	18.6	21.3
4	12.1	15.7
6	7.8	10.4
8	3.2	5.6

Statistical Analysis

Statistical Parameter	Healthy Group	Fever Group
Mean Metabolite Intensity	10.6	14.8
Standard Deviation	2.1	3.4
Variance	4.41	11.56
Standard Error	0.66	1.07
p-Value	<0.01	<0.001

The graphical data clearly indicate significant metabolic differences between healthy and fever-induced plasma samples. Pie chart analysis showed reduced glucuronidation and sulfation in fever conditions, whereas oxidative metabolite formation increased considerably.

Bar graph comparison demonstrated elevated cysteine conjugates and oxidative metabolites in fever plasma samples, suggesting enhanced detoxification of reactive intermediates. The line graph revealed slower clearance and altered plasma concentration patterns during fever conditions.

Statistical analysis confirmed that the observed metabolic variations were significant, supporting the hypothesis that fever and inflammatory stress alter paracetamol metabolism.

The present study successfully demonstrated the application of LC-HRMS for comparative profiling of paracetamol metabolites in healthy and fever-induced human plasma samples. HRMS provided accurate detection of both major and minor metabolites with high sensitivity and mass precision.

In healthy individuals, paracetamol metabolism mainly proceeded through glucuronidation and sulfation pathways, which are considered safe detoxification mechanisms. High levels of paracetamol glucuronide and sulfate metabolites confirmed normal hepatic metabolic activity.

In contrast, fever-induced plasma samples showed reduced glucuronidation efficiency and increased production of oxidative metabolites. The elevated concentration of cysteine conjugates and mercapturic acid derivatives suggests increased detoxification of reactive intermediates generated during oxidative metabolism.

The increased abundance of NAPQI-related metabolites in fever conditions indicates that inflammatory stress may influence cytochrome P450-mediated oxidation pathways. Such alterations may increase the risk of hepatotoxicity if high doses of paracetamol are administered during prolonged fever or infection.

The findings of this study are consistent with previous pharmacometabolomic investigations reporting disease-associated changes in drug metabolism. HRMS-based metabolite profiling can therefore serve as an effective analytical approach for understanding physiological influences on drug biotransformation.

Overall, the study highlights the importance of monitoring metabolic variations under pathological conditions for improving drug safety, therapeutic monitoring, and personalized medicine approaches.

CONCLUSION

The present study successfully demonstrated the comparative HRMS profiling of paracetamol metabolites in healthy and fever-induced human plasma samples. Significant differences were observed in the metabolic behavior of paracetamol under normal and fever-associated physiological conditions.

Healthy plasma samples mainly showed higher levels of glucuronide and sulfate conjugates, indicating normal detoxification pathways. In contrast, fever-induced samples exhibited increased oxidative metabolites, cysteine conjugates, and mercapturic acid derivatives, suggesting altered hepatic metabolism and elevated oxidative stress. The findings confirm that fever and inflammatory conditions can influence paracetamol biotransformation pathways and may increase the formation of reactive intermediates. Such metabolic alterations may affect drug efficacy and toxicity during disease conditions.

High-Resolution Mass Spectrometry proved to be a highly sensitive and reliable analytical technique for metabolite identification and comparative plasma profiling. The technique enabled accurate detection of both major and trace metabolites with excellent mass accuracy.

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