CHAPTER 6

In-Vitro Studies
SOLUBILITY ASSAY IN PHOSPHATE BUFFER SALINE:- (KINETIC METHOD)

OBJECTIVE’S

The goal of the study was to perform dissolvability examine in phosphate support saline (active system) utilizing HPLC technique for evaluation of solvency of Tolbutamide.

Solubility:- Solubility determination is measure of solvent capacity to dissolve the solute. For conduct of any study, we need to spike a required concentration of compound in desired medium and a measure of solubility ascertains that level of compound concentration can be added/maintained in assay conditions. Dynamic solvency is commonly a measure of compound dissolvability in watery media when included from a concentrated stock (in DMSO).

MATERIAL AND METHODS

1.1 Test substance information:-

<table>
<thead>
<tr>
<th>Test substance Name</th>
<th>:-</th>
<th>Tolbutamide</th>
</tr>
</thead>
<tbody>
<tr>
<td>Chemical Name (IUPAC)</td>
<td>:-</td>
<td>1-Butyl-3-(4-methylphenylsulfonyl) urea</td>
</tr>
<tr>
<td>Batch No.</td>
<td>:-</td>
<td>011M1397V</td>
</tr>
<tr>
<td>CAS No.</td>
<td>:-</td>
<td>64-77-7</td>
</tr>
<tr>
<td>Manufactured by</td>
<td>:-</td>
<td>Sigma Aldrich Company 3050 , Spruce saint Louis, MO 63103 USA</td>
</tr>
<tr>
<td>Production Date</td>
<td>:-</td>
<td>January 2011</td>
</tr>
<tr>
<td>Expiry date</td>
<td>:-</td>
<td>January 2017</td>
</tr>
<tr>
<td>Purity</td>
<td>:-</td>
<td>99.7 % (As per COA)</td>
</tr>
<tr>
<td>Storage Condition</td>
<td>:-</td>
<td>Room temperature (20-25°C)</td>
</tr>
<tr>
<td>Test substance procurement</td>
<td>:-</td>
<td>Sigma Aldrich</td>
</tr>
</tbody>
</table>
1.2 Apparatus

- Micro balance, having readability ± 0.01 mg, (Sartorius; Model ME-5)
- Semi Micro balance, (Sartorius; Model C225D)
- Waters Alliance High Performance Liquid Chromatograph equipped with UV and PC based data system with Empower Software (Waters Corporation, U.S.A.).
- HPLC Column: - Symmetry sheild, RP 18, 5 µ, 250 X 4.6 mm, Waters
- Ultra-sonicator, Make: - S40H, Elma sonic, Ireland
- Filtration unit, Make: - Millipore, X10422050
- Vortexer, Make: - Spinix
- 0.22 µm membrane filter, Make: - Millipore, Durapore, GVNP
- pH meter, Make: - LabIndia
- Refrigerator, Make: - VESTFROST, Model: - CFK471
- Magnetic stirrer, Make: - SPINOT

1.3 Labware

- Multi pulse vortexer: - Glas-Col
- Vacuum pump
- 96-well multi-screen filter plate: - Millipore
- 96-well acceptor plate: - Nunc
- Manifold filtration assembly: - Millipore
1.4 Chemicals used:

- Acetonitrile, HPLC grade, Biosolv, Batch no. 901911 and Expiry date: 5 Sep 2015
- Ammonium Acetate, Sigma Aldrich, Batch no. STBB6733V and Expiry date: 14 Aug 2018
- Acetic Acid, AR Grade, Rankem, Batch no. R023H06 and Expiry date: 24 Aug 2015
- Methanol, HPLC grade, Biosolv, Batch no. 944761 and Expiry date: 14 Jan 2016
- Dimethylsulfoxide (DMSO) SD fine chemicals Limited, Batch no. 56011J05 and Expiry date: 1 Jan 2020
- Phosphate Buffer Saline (PBS) Sigma Aldrich and Expiry date: 1 Jan 2020
- Potassium dihydrogen phosphate (KH$_2$PO$_4$) Sigma Aldrich and Expiry date: 1 Jan 2020
- Di-Potassium hydrogen phosphate (K$_2$HPO$_4$) Sigma Aldrich and Expiry date: 1 Jan 2020

METHODOLOGY

- Approximately 2 mg of the tolbutamide was weighed and dilute appropriately to attain 20 mM DMSO stock solution.

- Seven level calibration standards (i.e. 5, 10, 25, 50, 100, 200 and 300 µM) in DMSO were made from the above DMSO stock solution (20 mM).

- To multiscreen solubility filter plate; 198 µL of PBS buffer was dispensed into duplicate wells, 2 µL of test compound (20 mM DMSO stock) was added, plate was sealed and shaken at 150 rpm for 90 min.

- Samples were filtered using Manifold filtration assembly and filtrate was collected in acceptor plate.

- 150 µL of filtrate from 96-well acceptor plate (tolbutamide) and standards were transferred to HPLC vials and analyze by HPLC-UV.
RESULTS

Calibration curve was plotted for a CC range of 0.9 to 270 µM. Regression coefficient obtained for calibration curve was 0.99108 with all seven standard points with % deviation between -14.7 to 8.893.

Solubility value obtained was 198.9 µM i.e. equivalent to 53.77 µg/mL. Obtained value indicated good aqueous solubility of tolbutamide.

REFERENCES

- Kirns EH and Di Li.;(2005) “Automation in Pharmaceutical Profiling; Journal of the Association for Laboratory Automation”; vol -10 page - 114-123.

FIGURE-1: CC plot for solubility assay

FIGURE-2: CC Back calculated values
FIGURE-3: Solubility values for spiked samples

<table>
<thead>
<tr>
<th>Name</th>
<th>Level</th>
<th>X Value</th>
<th>Response</th>
<th>Calc. Value</th>
<th>% Deviation</th>
<th>Manual</th>
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<td>TOLBUTAMIDE_PBS</td>
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<td>112204.79305</td>
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<td>TOLBUTAMIDE_PBS</td>
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<td>187658.536615</td>
<td>8.101712</td>
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<td>TOLBUTAMIDE_PBS</td>
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<td>901316.81665</td>
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<td>TOLBUTAMIDE_PBS</td>
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<td>4942574.842346</td>
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<td>2.505</td>
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Component Summary Table
**Sample Type: Unknown**

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<th>SampleName</th>
<th>Sample Type</th>
<th>Vial</th>
<th>Retention Time (min)</th>
<th>Area</th>
<th>Amount</th>
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<tr>
<td>TOLBUTAMIDE_PBS-T-1</td>
<td>Unknown</td>
<td>89</td>
<td>4.738</td>
<td>3563174</td>
<td>198.825</td>
<td>µM</td>
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<tr>
<td>TOLBUTAMIDE_PBS-T-2</td>
<td>Unknown</td>
<td>90</td>
<td>4.727</td>
<td>3566009</td>
<td>198.985</td>
<td>µM</td>
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<tr>
<td>Mean</td>
<td></td>
<td></td>
<td></td>
<td>356459.2</td>
<td>198.9</td>
<td></td>
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<tr>
<td>Std. Dev.</td>
<td></td>
<td></td>
<td></td>
<td>2005</td>
<td>0.1</td>
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ASSESSMENT OF METABOLIC STABILITY IN POOLED LIVER MICROSONES

OBJECTIVE

The goal of the study was to focus the metabolic degradation-rate of a tolbutamide in pooled liver microsomes. The metabolic stability determination utilizing liver microsomes is invitro model to gauge hepatic metabolism. This model has been generally used to acquire early forecast of hepatic leeway for compound, quickening the recognizable proof of lead mixes.

SUBTANCE AND METHODS

1.1 equipment:-

- Atlantis dC18, 4.6 X 50 mm, 3 µ
- HPLC System – Shimadzu Nexera
- Mass Spectrometer – Applied Biosystems, MDS Sciex, API-4000 QTrap
- Deep Freezer – Thermo Electron Corporation
- Freezer – Vestfrost
- Refrigerator – Vestfrost
- Semi Micro Balance – Sartorius CP225D
- Microbalance – Sartorius ME5
- Vortexer – Spinix
- Water purification System – Milli-Q, Millipore
- Micropipettes and multi-pipettes – Eppendorf
- Refrigerated centrifuge – Eppendorf 5430R
- Ultra-sonicator , Make:-S40H, Elma sonic, Ireland
1.2 Chemicals used

- Tolbutamide (Reference standard, Batch no 011M1397V, Sigma-Aldrich)
- Acetonitrile (HPLC grade, Lot no. 957651 JT Baker)
- Methanol (HPLC grade, Batch no.944761 JT Baker)
- Formic acid (Puriss, Batch no. 3270479 Spectrochem)
- Type 1 Water (Milli-Q Water purification System)
- β-Nicotinamide adenine dinucleotide-2 phosphate, Sigma Aldrich
- Dimethyl sulfoxide (DMSO) Sigma Aldrich
- Dipotassium hydrogen phosphate (K2HPO4) Sigma Aldrich
- Potassium dihydrogen phosphate (KH2PO4)
- Pooled liver microsomes

PREPARATION OF REAGENTS/SOLUTIONS

Buffer preparation

- 1 M K2HPO4 (MW=174.18) :- Dissolved 17.4 g of K2HPO4 in 100 milli liter of Milli-Q water.
- 1 M KH2PO4 (MW=136) :- Dissolved 13.6 g of KH2PO4 in 100 milli liter of Milli-Q water.
- 0.1 M Potassium phosphate buffer:- Mixed 8.02 mL of 1 M K2HPO4 and 1.98 mL of 1 M KH2 PO4 and diluted the quantity to 100 mL with Milli-Q.
• 66.7 mM Potassium phosphate buffer (PPB, pH=7.4) :- Diluted 66.7 mL of 0.1 M potassium phosphate buffer with Milli-Q water up to 100 mL.

NADPH (10 mM) preparation
• Weighed 8.33 mg of NADPH and dissolved in 1 mL PPB to get a 10 mM solution.

Reference standards and test compounds stock solution preparation
• 20 mM (reference standard/test compound) (MW = 270) :- Weighed 5.4 mg of tolbutamide and dissolved in 1 mL of DMSO to obtain 20 mM stock solution.
• 1 mM (reference standard/test compound) :- Took 50 µL of the above 20 mM stock solution and diluted to 1 mL in DMSO to get 1 mM operational stock solution.

Quenching solution preparation
• 0.5% Formic acid in internal standard (IS) containing ACN: 5 mL of formic acid was added in 1 L of ACN.

METHODOLOGY
• Vials containing microsomes were removed from -70 ± 10°C deep freezer and thawed on the surface of ice bath.
• 27.5 µL microsomes (20 mg/mL) was suspended in ~971.5 µL of 66.7 mM potassium phosphate buffer in the polypropylene tube in order to get an approximate concentration of 0.5 mg/mL of microsomal protein.
• 1.1 µL of tolbutamide or manage (1 mM) in the reaction mixture was added and labeled as incubation mixture.
• 180 µL of aliquots were taken from incubation mixture and labeled as Tc, T0, T5, T15 and T30.
• Pre-incubated all the tubes at 37 ± 1°C for 5 min in shaking water bath along with the test compound/reference standard.

• After pre-incubation, 20 µL of NADPH (10 mM) was added in all tubes (T0, T5, T15 and T30) and kept for incubation.

• After pre-incubation, 20 µL of potassium phosphate buffer (instead of NADPH) was added to the tube labeled Tc (control incubation) and incubate for 30 min.

• At the end of the incubation period (Tc, T0, T5, T15 and T30) of respective tubes, 200 µL of quenching solution was added to each vial to arrest the reaction.

• Ensuing sample were centrifuged at 4000 rpm for 10 min.

• 200 µL supernatant from each vial was transferred into HPLC vial for the analysis on LC-MS/MS.

CALCULATION

Calculation of microsomal intrinsic clearance (mCLint) was based on the substrate (test compound) disappearance rate. The chromatographic peaks were integrated (using Analyst® from Applied Biosystem Sciex). The resultant peak area ratios between test compound/reference standard and ISTD were used for the calculations. A linear plot of natural logarithm (Ln) area vs time was created to visualize the data. The slope was calculated for each data set. Subsequently, half life (T1/2) and mCLint were considered according to the following equations:

Percent metabolized: - \[1-(test \ area \ ratio/control \ area \ ratio)\] \*100

\[T_{1/2} = \frac{\ln 2}{-\text{slope}}\]

\[m,CL_{int} = \frac{\ln 2 \times 1000}{T_{1/2} (min) \times proteinconc (mg/ml)}\]

\[\ln 2 = 0.693\]
RESULTS

Percent vanishing was generally all the more in Human Liver microsomes, when contrasted with other 2 species tried, showing a Human particular isoenzyme majorly in charge of digestion system of medication. Half life in human liver microsomes was most minimal (i.e. 90 minutes) when contrasted with other 2 species (i.e. Rodent and Mouse) ~ 180 minutes. mCLint values (1.0) for human liver microsomes were in close concurrence with published (Obach; 1999).

REFERENCES

• Dermet F. and Mcginity l, Robrnt J. Reley et.al;(2004) ; Evaluation of new and cryopreserved hepatocytes as in vitro drug digestion system apparatuses for the expectation of metabolic freedom; Drug Metab Dispos vol.-32;pp.-1246-1254.


• Austen RP, Mcginity DF, and Reley RJ (2005) A brought together model for anticipating human hepatic, metabolic leeway from in vitro inborn freedom information in hepatocytes and microsomes. Drug Metab Dispos 33:- 1302-13010
ASSESSMENT OF CYTOCHROME P450 INHIBITION IN POOLED HUMAN LIVER MICROSONMES

OBJECTIVE

The objective of this study was to determine the cytochrome P450 (CYP450) inhibition by tolbutamide in pooled human liver microsomes (HLM).

The in-vitro pooled human liver microsomes CYP450 inhibition assay is used to evaluate the potential of compounds to cause drug-drug interactions, through inhibition of clinically important CYP450 isoforms. Co-incubation of the probe substrate with the test inhibitor allows the calculation of percent inhibition/IC50 measurement. A decrease in the formation of the metabolite(s) compared to the vehicle control is used to calculate the inhibition/IC50 value.

MATERIAL AND METHODS

1.1 Apparatus

- Atlantis dC18, 4.6 X 50 mm, 3 µ
- HPLC System – Shimadzu Nexera
- Mass Spectrometer – Applied Biosystems, MDS Sciex, API-4000 QTrap
- Deep Freezer – Thermo Electron Corporation
- Freezer – Vestfrost
- Refrigerator – Vestfrost
- Semi Micro Balance – Sartorius CP225D
- Microbalance – Sartorius ME5
- Vortexer – Spinix
- Water purification System – Milli-Q, Millipore
- Micropipettes and multi-pipettes – Eppendorf
- Refrigerated centrifuge – Eppendorf 5430R
- Ultra-sonicator, Make:-S40H, Elma sonic, Ireland

1.2 Chemicals used

- Tolbutamide (Reference standard, Batch no 011M1397V, Sigma-Aldrich)
- Acetonitrile (HPLC grade, Lot no. 957651  JT Baker)
- Methanol (HPLC grade, Batch no.944761 JT Baker)
- Formic acid (Puriss, Batch no. 3270479  Spectrochem)
- Type 1 Water (Milli-Q Water purification System)
- β-Nicotinamide adenine dinucleotide-2 phosphate , Sigma Aldrich
- Dimethyl sulfoxide (DMSO)  Sigma Aldrich
- Dipotassium hydrogen phosphate (K2HPO4)  Sigma Aldrich
- Potassium dihydrogen phosphate (KH2PO4)
- Pooled liver microsomes

**PROBE SUBSTRATES**

<table>
<thead>
<tr>
<th>CYP Isoform</th>
<th>Probe substrate</th>
<th>Catalogue no.</th>
<th>Manufacturer</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A4</td>
<td>Midazolam</td>
<td>BP98/EP2000</td>
<td>Hangzhou Pharma</td>
</tr>
<tr>
<td>2D6</td>
<td>Bufuralol hydrochloride</td>
<td>B689540</td>
<td>BD Gentest</td>
</tr>
<tr>
<td>2C9</td>
<td>Diclofenac</td>
<td>DS-6102</td>
<td>Cyprotex</td>
</tr>
<tr>
<td>2C19</td>
<td>Omeprazole</td>
<td>520001</td>
<td>Cypex</td>
</tr>
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</table>

**REFERENCE INHIBITORS**

<table>
<thead>
<tr>
<th>CYP Isoform</th>
<th>Inhibitor</th>
<th>Catalogue no.</th>
<th>Manufacturer</th>
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<tbody>
<tr>
<td>3A4</td>
<td>Ketoconazole</td>
<td>K1003</td>
<td>Sigma</td>
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<tr>
<td>2D6</td>
<td>Quinidine</td>
<td>Q3625</td>
<td>Sigma</td>
</tr>
<tr>
<td>2C9</td>
<td>Sulphaphenazole</td>
<td>S0758</td>
<td>Sigma</td>
</tr>
<tr>
<td>2C19</td>
<td>Nootkatone</td>
<td>1326499</td>
<td>Fluka</td>
</tr>
</tbody>
</table>

**PREPRATION OF REAGENTS/ SOLUTIONS**
Buffer preparation

- 1 M K2HPO4 (MW=174.18) :- 17.4 g of K2HPO4 was broken down in 100 milliliter of Milli-Q water.

- 1 M KH2PO4 (MW=136) :- 13.6 g of KH2PO4 was broken down in 100 milliliter of Milli-Q water.

- 0.1-M Potassium phosphate Soln.: 8.02 mL of 1 M K2HPO4 and 1.98 mL of 1 M KH2PO4 were added and weakened up to 100 mL with Milli-Q water.

- 66.7 mM Potassium phosphate Soln. (PPB, pH=7.4) :- 66.7 mL of 0.1 M potassium phosphate soln. was weakened with Milli-Q water up to 100 mL.

NADPH (10 mM) preparation

- 8.33 mg of NADPH was dissolved in 1 mL Potassium phosphate buffer to get a 10 mM solution.

Quenching solution preparation

- 0.5% Formic acid in internal standard (IS) containing ACN:- 5 mL of formic acid in 1 L of ACN containing suitable ISTD.

METHODOLOGY

Preparation of incubation mixture

- Microsomes were thawed by keeping on ice for 10 minutes.

- Microsomal suspension in potassium phosphate buffer was prepared, to yield the required final protein concentration.
• Probe substrate were added to the above microsomal suspension to get a final concentration as defined in the below table.

• Contents were mixed gently by inverting and equilibrating at 37 ± 10°C for 5 min.

**Experimental procedure**

• 801 µL of the above incubation mixture was added into three polypropylene tubes labeled “Test”, “Test control” and “Positive control”.

• 0.9 µL of the test compound stock solution (final concentration in the reaction mixture: - 2 and 20 µM) was added to the tube labeled “Test”.

• 0.9 µL of DMSO was added to the tube labeled “Test control”.

• 0.9 µL of reference inhibitor solution was added to the tube labeled “Positive control”.

• Incubation mixture was mixed properly.

• Two aliquots (270 µL x 2) of each of the above incubation mixtures were taken into labeled tubes.

• All tubes were incubated in shaking water bath at 37 ±1°C for 5 min.

• Reactions were initiated by adding 30 µL of 10mM NADPH to all samples and incubate at 37 ±1°C for 10 min.

• After completion of incubation time vials were removed from shaking water bath.

• After completion of incubation time, reactions were stopped by adding 300 µL of quenching solution.

• Tubes were gently vortexed and centrifuged at 3,100 rpm for 20 min at 40°C.

• Samples were analysed on LC-MS/MS system.

• Metabolite formation for each substrate under below conditions was estimated employing LC-MS/MS system.
REACTION CONDITIONS

<table>
<thead>
<tr>
<th>CYP450 isoform</th>
<th>Probe substrate</th>
<th>Activity</th>
<th>Final substrate conc. (µM)</th>
<th>Protein conc. (mg/mL)</th>
<th>Positive control conc. (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>3A4</td>
<td>Midazolam</td>
<td>1’-Hydroxylation</td>
<td>2.5</td>
<td>0.1</td>
<td>0.037</td>
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<td>2D6</td>
<td>Bufuralol</td>
<td>1’-Hydroxylation</td>
<td>5</td>
<td>0.25</td>
<td>0.5</td>
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<td>2C9</td>
<td>Diclofenac</td>
<td>4’- Hydroxylation</td>
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<td>Omeprazole</td>
<td>5 - Hydroxylation</td>
<td>20</td>
<td>0.5</td>
<td>300</td>
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- **Incubation period**: 10 min

CALCULATION

Percent inhibition = \[1-(\text{test area ratio/控制 area ratio})\]*100

ACCEPTANCE CRITERIA FOR CONTROLS

<table>
<thead>
<tr>
<th>CYP Isoform</th>
<th>Probe substrate</th>
<th>Positive control</th>
<th>Acceptance range (% inhibition)</th>
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<tr>
<td>3A4</td>
<td>Midazolam</td>
<td>Ketoconazole</td>
<td>65-75</td>
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<tr>
<td>2D6</td>
<td>Bufuralol hydrochloride</td>
<td>Quinidine</td>
<td>80-90</td>
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<td>2C9</td>
<td>Diclofenac</td>
<td>Sulphaphenazole</td>
<td>60-70</td>
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<tr>
<td>2C19</td>
<td>Omeprazole</td>
<td>Nootkatone</td>
<td>65-75</td>
</tr>
</tbody>
</table>
RESULTS

- **Isoenzyme 3A4:-** No significant inhibition was seen at lower concentration level tested, while % inhibition for higher concentration level was ~ 7%. Percent inhibition obtained for control (Ref. inhibitor i.e. Ketoconazole) was 73%.

- **Isoenzyme 2D6:-** Inhibition observed was ~ 13 and 51% at lower and higher concentration levels tested. Percent inhibition obtained for control (Ref. inhibitor i.e. Quinidine) was 85%.

- **Isoenzyme 2C9:-** No significant inhibition was seen at lower concentration level tested, while % inhibition for higher concentration level was ~ 4%. Percent inhibition obtained for control (Ref. inhibitor i.e. Sulphaphenazole) was 65%.

- **Isoenzyme 2C19:-** No significant inhibition observed at both lower and higher concentration levels tested. Percent inhibition obtained for control (Ref. inhibitor i.e. Nootkatone) was 64%.

REFERENCES

- Meia Turpenen, Rina Neminen, Tarija Jantunen, Päivi Tavitsainen1, Hanu Raunieo and Olaiv Pelknen (2004) "Particular hindrance of cyp2b6-catalyzed bupropion hydroxylation in human liver microsomes in-vitro, Drug Metabolism and Dispos" vol.-32no.-(6); pp.-625-632.


• Mankwaski DC (1999) "The part of CYP2C19 in the digestion system of (+/−) bufuralol, the prototypic substrate of CYP2D6. Drug Metab Dispos" vol.-27;pp.-1022–1026.

ASSESSMENT OF PLASMA PROTEIN BINDING (EQUILIBRIUM DIALYSIS METHOD)

OBJECTIVE

The objective of this study was to determine the plasma protein binding of tolbutamide in plasma aliquots via equilibrium dialysis method.

The extent of drug binding to plasma proteins influences the way drug distributes in body tissues. Fraction unbound for plasma binding is an indicator of higher proportion of free drug available for pharmacological action. Plasma protein binding was determined by equilibrium dialysis method.

MATERIAL AND METHODS

1.1 Apparatus

- Atlantis dC18, 4.6 X 50 mm, 3 µ
- HPLC System – Shimadzu Nexera
- Mass Spectrometer – Applied Biosystems, MDS Sciex, API-4000 QTrap
- Deep Freezer – Thermo Electron Corporation
- Freezer – Vestfrost
- Refrigerator – Vestfrost
- Semi Micro Balance – Sartorius CP225D
- Microbalance – Sartorius ME5
- Vortexer – Spinix
- Water purification System – Milli-Q, Millipore
- Micropipettes and multi-pipettes – Eppendorf
- Refrigerated centrifuge – Eppendorf 5430R
- Ultra-sonicator , Make:-S40H, Elma sonic, Ireland

1.2 Chemicals used

- Tolbutamide (Reference standard, Batch no 011M1397V, Sigma-Aldrich)
- Acetonitrile (HPLC grade, Lot no. 957651 JT Baker)
Methanol (HPLC grade, Batch no.944761 JT Baker)
Formic acid (Puriss, Batch no. 3270479 Spectrochem)
Type 1 Water (Milli-Q Water purification System)
Dimethyl sulfoxide (DMSO) Sigma Aldrich Rat Plasma
HT-Dialysis® 96-well plate, Gales Ferry, CT, USA (HT-Dialysis, Gales Ferry, CT, USA, Prod# 1101) 12-14 kD molecular weight cut off

METHODOLOGY

- Membrane was soaked in Milli-Q water for 60 min and subsequently by 20% ethanol in Milli-Q water for 30 min. Membrane was rinsed well with Milli-Q water twice before loading on HT dialyzer.
- 150 µL plasma (n=3) aliquots containing 3 µM final drug concentration were added in first half of the well of the 96-well Micro-Equilibrium Dialysis Device HTD.
- 150 µL (n=3) of blank sodium phosphate buffer aliquots were added in the buffer half of the same well of the above plate.
- Plate was equilibrated at 37 ± 5°C for 4.5 h, with constant rotation at 130 rpm on an orbital shaker.
- After equilibration time was over, 10 µL of plasma sample was taken from the first half of the well to a vial/cover contain 200 µL of acetonitrile and 50 µL of blank buffer was also added.
- Similarly 50 µL of buffer sample was taken from the buffer half of the wells to a vial containing 200 µL acetonitrile and 10 µL of blank plasma was also added.
- All samples were centrifuged at 14,000 rpm for 5 min.
- Supernatant was transferred to LC-MS/MS vials.
- For recovery samples 10 µL of plasma aliquot from plasma sample containing 3 µM of final drug concentration was added to a vial containing 200 µL of acetonitrile and 50 µL of blank buffer was also added to same vial.
CALCULATION

Fraction unbound for plasma binding was calculated using the following equation:-

\[
\frac{[\text{Buffer}/5]}{[\text{Plasma}]}
\]

\[\text{fu} = \frac{[\text{Buffer}/5]}{[\text{Plasma}]},\]

Where \([\text{plasma}]\) is the conc. calculated in the plasma and \([\text{buffer}]\) the conc. calculated in the buffer. Buffer conc. have to be divided by factor 5, since five times higher volume of buffer is used for concentration determination.

RESULTS

Fraction unbound value obtained was 0.04, 0.05 and 0.04 for 10, 50 and 100 % plasma experiments, respectively. Values obtained were in close agreement with published values and indicate high protein binding property for tolbutamide.

REFERENCES

ASSESSMENT OF MDR1 TRANSFECTED MDCK PERMEABILITY ASSAY

OBJECTIVE:-

The target of the study was to assess penetrability of tolbutamide through MDR1 transfected MDCK cells. P-glycoprotein (P-gp) is most broadly concentrated on medication transporter because of its potential part in medication aura and adequacy, and medication drug communications (DDI). It is plentifully communicated in both the intestinal divider and blood-cerebrum obstruction where it serves as a medication porousness hindrance while at the same time encouraging medication disposal in the liver and kidney. It is additionally inexhaustibly communicated in tumors where it can encourage the disposal of chemotherapeutics, a sensation known as multidrug resistance (MDR). Penetrability of medication crosswise over Madin Darby canine kidney (MDCK) monolayer (MDR1 transfected) is a measure of its oral retention and demonstrative of its instrument of transport. Evident porousness of test compound from the basal to apical and apical to basal side of the cell monolayer will be resolved for efflux potential outcomes. {C L Laam K et.al. (2012)}.

Permeability study was conducted with the MDR1 transfected MDCK monolayers cultured for 3 days. Aliquots of 100 µL sample from apical and basolateral chambers at 0 and 60 min time points were quenched. The appearance of area counts for tolbutamide vs its area in preliminary chamber at initial time was considered to determine rate of permeability. Determination of convey in both directions (apical to basal (A-B) and basal to apical (B-A) ) crossways the cell monolayer enabled an efflux ratio to be calculated which provided an indicator as to whether a compound undergoes active efflux can be likely for tolbutamide.

MATERIAL AND METHODS

1.1 Apparatus

- Atlantis dC18, 4.6 X 50 mm, 3 µ
- HPLC System – Shimadzu Nexera
- Mass Spectrometer – Applied Biosystems, MDS Sciex, API-4000 QTrap
- Deep Freezer – Thermo Electron Corporation
- Freezer – Vestfrost
• Refrigerator – Vestfrost
• Semi Micro Balance – Sartorius CP225D
• Microbalance – Sartorius ME5
• Vortexer – Spinix
• Water purification System – Milli-Q, Millipore
• Micropipettes and multi-pipettes – Eppendorf
• Refrigerated centrifuge – Eppendorf 5430R
• Ultra-sonicator, Make:-S40H, Elma sonic, Ireland

1.2 Chemicals used
• Tolbutamide (Reference standard, Batch no 011M1397V, Sigma-Aldrich)
• Acetonitrile (HPLC grade, Lot no. 957651  JT Baker)
• Methanol (HPLC grade, Batch no.944761 JT Baker)
• Formic acid (Puriss, Batch no. 3270479  Spectrochem)
• Type 1 Water (Milli-Q Water purification System)
• 0.05% Trypsin-EDTA, Sigma Aldrich
• 24-well Millicell culture plate, Millipore
• 4-(2-Hydroxyethyl) -1-piperazineethanesulfonic sour Sigma Aldrich
• Dulbecco’s customized Eagle Media
• Fetal bovine serum (FBS)
• Hanks balanced saline solution (HBSS)

METHODOLOGY
Permeability study was conducted with the MDR1 transfected MDCK monolayers cultured for 3 days.

Trypsinize and centrifuged approximately 80-90% confluent MDR1 transfected MDCK cells.

Resuspended the pellet in fresh media and counted the cells using hemocytometer.

500 µL aliquot of above suspension (comprising 5,00,000 cells/well) was loaded onto 24-well Millicell plate (apical chamber, A) and 18 mL of media was added to the basal tray.

Culture media was replace every 2 days by maintain the cells at 37 ± 1°C with 5% CO₂.

3-4 day cultured MDCK/MDR1 cell monolayer with average TEER values of 600-800 Ohm x cm² was taken for experiments.

MDCK/MDR1 Study Protocol (A to B)

HBSS buffer containing 10 mM HEPES was used as transport media for permeability study.

Prior to experiment, monolayer was washed twice with HBSS buffer.

Apical plate was placed on the 24-well transporter plates.

1 mL of HBSS was added to the respective basolateral wells.

Permeability study was initiated by adding 0.3 mL of test compound to apical chamber (n=2).

Incubated the plate setup on a shaking incubator at 37 ± 1°C with 100 rpm.

100 µL sample were aliquoted from apical and basolateral chambers at 0 and 60 min time points.
• Samples were extinguished with 100 µL of extinguishing arrangement (i.e. ACN with ISTD).
• Samples were centrifuged for 5 minutes at 3000 rpm and supernatant aliquote was subjected for investigation by LC-.

**MDCK/MDR1 Study Protocol (B to A)**

• Prior to experiment, monolayer was washed twice with HBSS buffer.
• Apical plate was placed on the 24-well transporter plates.
• 1 mL of test compound was added in their respective basolateral wells (n=2).
• Permeability study was initiated by adding 0.3 mL of HBSS buffer to apical chamber (n=2).
• Incubated the plate setup on a shaking incubator at 37 ± 1°C with 100 rpm.
• 100 µL samples were aliquoted from apical and basolateral chambers at 0 and 60 min time points.
• Taster was quenching with 100 µL of quenching solution (i.e. ACN with internal standard).
• Samples were centrifuged for 5 minutes at 3000 rpm and supernatant aliquote was subjected for psychoanalysis by LC-MS/MS.

**CALCULATION**

\[ P_{\text{app}} = \frac{V_{r}}{C_{0}} \left( \frac{1}{S} \right) \left( \frac{dC}{dt} \right) \]

\( P_{\text{app}} = \) app. permeability, \( V_{r} = \) volume of medium in the receiver chamber, \( C_{0} = \) concentration of the test drug in the donor chamber, \( S = \) surface area of monolayer, \( dC/dt = \) drug concentration in the receptor chamber with time).
Area of 24-well = 0.7 cm$^2$

Efflux ratio (ER) = $\frac{P_{B \to A}}{P_{A \to B}}$

($P_{B \to A}$ and $P_{A \to B}$ represent the apparent permeability of compound from the basal to apical and apical to basal sides across the cellular monolayer).

Recovery for the experiment was calculated using below formula:-

\[
\text{Recovery} [\%] = \left[ \frac{(QD_{\text{end}} + QR_{\text{end}})}{QD_{\text{0 min}}} \right] \times 100
\]

\[(QD_{\text{end}} + QR_{\text{end}}) \text{ is the final conc. of compound remaining in both donor and receiver sections. (QD}_{\text{0 min}} \text{ is the amount in the donor side at start (0 min))}\]

RESULTS

Apparent permeability observed for tolbutamide was $58.43 \times 10^{-6}$ cm/sec for apical to basal (A-B) trial and $23.35 \times 10^{-6}$ cm/sec for basal to apical (B-A) experiment. Efflux ratio obtained was 0.40, indicating absence of any significant efflux for tolbutamide.

Digoxin was used as control for mentioned experiment and apparent permeability observed was $0.86 \times 10^{-6}$ cm/sec for apical to basal (A-B) experiment and $18.4 \times 10^{-6}$ cm/sec for basal to apical (B-A) experiment. Efflux ratio obtained was 21.4.

REFERENCES