MATERIALS & METHODS
<table>
<thead>
<tr>
<th>No</th>
<th>Name</th>
<th>Grade</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Water for HPLC</td>
<td>Double distilled</td>
<td>-</td>
</tr>
<tr>
<td>7.</td>
<td>Diethyl ether</td>
<td>AR</td>
<td>TKM Pharma, Hyderabad.</td>
</tr>
<tr>
<td>8.</td>
<td>β-Glucuronidase</td>
<td>Type-1 from</td>
<td>Sigma Chemical CO, USA. Lot No. 88-H-3365</td>
</tr>
<tr>
<td></td>
<td>Helix pomatia</td>
<td></td>
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<tr>
<td>9.</td>
<td>Sodium acetate</td>
<td>AR</td>
<td>Qualigens Fine Chemicals, Mumbai</td>
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<tr>
<td>11.</td>
<td>Sodium carbonate</td>
<td>AR</td>
<td>Qualigens Fine Chemicals, Mumbai</td>
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<tr>
<td>12.</td>
<td>Hydrochloric acid</td>
<td>AR</td>
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<tr>
<td>13.</td>
<td>Orthophosphoric acid</td>
<td>AR</td>
<td>Qualigens Fine Chemicals, Mumbai</td>
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<tr>
<td>14.</td>
<td>Dextromethorphan</td>
<td>AR</td>
<td>i) Astra IDL India Ltd., Bangalore</td>
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<tr>
<td></td>
<td></td>
<td></td>
<td>ii) Boehringer Mannheim India Ltd., Maharashtra,</td>
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<td></td>
<td></td>
<td></td>
<td>ii) Dept of Clinical Pharmacology, University Hospital, Uppsal.</td>
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</table>
Reagents

1. Acetate buffer: pH-5

1.36g of sodium acetate and 0.6 ml of glacial acetic acid were dissolved in 100 ml distilled water. The pH was adjusted to 5 (if necessary) using sodium acetate or glacial acetic acid.

2. β-Glucuronidase:

One gram powder contains 3,74,000 units of β-glucouronidase. A mixture of 21.39 mg of the β-glucuronidase powder per ml of acetate buffer (pH 5) gave 8000U/ml of β-glucuronidase.

3. Saturated sodium carbonate solution:

29.6 g of sodium carbonate was dissolved in 200 ml of distilled water to make the saturated solution.

4. Solvent mixture for extraction:

Diethyl ether, chloroform and propan-2-ol mixed together in the ratio of 20 : 9 : 1 respectively. The mixture was frequently shaken during its use.

5. Hydrochloric acid (0.2 N)

1.72ml of concentrated HCl was added to 25 ml of double distilled water and the volume made up to 100 ml using double distilled water, in a volumetric flask.
Chromatography

Pump : Shimadzu LC-10AS
Injector : Rheodyne
Chromatopac integrator : C-R3A
Detector : RF-530 Fluorescent
Excitation wavelength : 230 nm
Emission wavelength : 330 nm
Column : Zorbax cyano column,
        5μm particle size (250 x 4.6mm).
Mobile phase : Methanol : Acetonitrile : Triethylamine (16:3:0.06%)
               in Double distilled water
pH : 2.8
Temperature : Ambient
Flow rate : 1ml/min
EXPERIMENTAL PROTOCOL

The study was approved by the ethical committee, Jawaharlal Institute of Post-graduate Medical Education and Research (JIPMER), Pondicherry.

Subjects:

A total of 454 South Indian healthy human volunteers participated in the study. The collection of samples was in collaboration with different organizations from the four South Indian states (Figure 10). The investigator visited all the four states personally and collected the samples with the help of the collaborators. The samples were transferred from the different states to the principle organization (JIPMER) in the frozen stage using dry ice.

The sample from Kerala was collected from staff and students of Department of Pharmaceutical Sciences, Mahatma Gandhi University, Kottayam, Kerala. In Karnataka the participants were the staff and students from Government College of Pharmacy, Bangalore, Krupanidhi College of Pharmacy, Bangalore, J.S.S. College of Pharmacy, Mysore and staff of Vetcare Pharmaceuticals, Bangalore, Karnataka. The participants of Tamil Nadu were from students of Perundurai Medical College and Research Center, Perundurai, Tamil Nadu and staff and students from JIPMER, Pondicherry. In Andhra Pradesh the participants were mostly the staff and students from Rengaraya Medical College, Kakinada, Andhra Pradesh.
Figure 10: The map of India with the location of sample collection for the present study.

1. Kottayam (Kerala)
2. Mysore (Karnataka)
3. Bangalore (Karnataka)
4. Perundurai (Tamil Nadu)
5. Pondicherry (Tamil Nadu)
6. Kakinada (Andhra Pradesh)
**Inclusion criteria:**

1. Unrelated subjects from either sex in the age group of 15-55 years.
2. Subjects whose parents are from the same South Indian state.
3. Subjects were judged to be in good health by a medical history and physical examination.
4. Subjects who gave their written concern for the participation in the study.

**Exclusion criteria:**

1. Subjects who were receiving any medication (including Ayurvedic drugs) on a chronic basis.
2. Subjects who were on concomitant therapy with drugs known to induce or inhibit the cytochrome P-450.
3. Chronic diseases.
5. Regular use of tobacco or alcohol.

**Study Protocol:**

The study protocol, its advantages and the possible side effects were explained to all the volunteers. Only those subjects who were voluntarily agreed for the study were recruited for the study. Special permission was also taken from the parents of subjects who were in the age group of 16-18 years old. All the volunteers gave their
written informed consent (Annexure-4). Personnel and medical history of each individual were noted in a pre-designed proforma (Annexure-5).

All the subjects received 30mg of dextromethorphan hydrobromide orally (5ml of Lactuss LA, cough suspension, FDC Ltd., Aurangabad, India) after emptying the bladder at bedtime. Overnight urine was collected for 8 hours. The total urine volume was recorded and about 15 ml aliquots were stored at -20°C until analysis. Side effects if any were noted.

The 30mg dose of dextromethorphan hydrobromide is corresponding to 23.1mg or 85µmol of dextromethorphan base. This value is used for all calculations.

**HPLC Analysis:**

**Extraction**

Dextromethorphan (DM) and its metabolite dextrorphan (DT) were extracted from the urine samples using the following extraction procedure\(^8\).

Samples were taken from the deep freezer and thawed at room temperature for 30 minutes. An aliquot of 0.5 ml of urine sample to be analyzed was transferred into a polypropylene tube. To cleave the glucuronide conjugation, each urine sample was incubated with 0.5ml β-glucuronidase (8000 units/ml at pH 5) for at 37°C for 17
hours. Standards were also prepared by spiking 100 and 250 ng of DM and 250
and 1000 ng of DT in 0.5ml drug free urine samples during each day of analysis.
From this point onwards standards and samples were treated identically.

After cooling down to room temperature, 250µl of saturated sodium carbonate was
added two times to each tube, to make the contents alkaline. The tubes were
vortexed for 10 seconds. Five milliliters of the organic extractant (20:9:1 of diethyl
ether:chloroform:2-propanol) was added, and each tube was shaken for 15 minutes.
The tubes were then centrifuged at 2,000 rpm for 15 minutes. The organic (top)
layer was then aspirated and placed into a 12 ml glass conical tubes which contained
400µl of 0.2N HCl. These conical tubes were vortexed for 60 seconds, then
centrifuged at 2,000 rpm for 10 minutes. The upper organic layer was removed
carefully. About 200µl of the acid layer was injected in to the HPLC column.

In the HPLC analysis, the retention time for DT and DM were approximately 4.8
and 7.6 minutes respectively. The recovery of DT was >80 and for DM it was
>70%. The inter-day and intra-day coefficient of variation for assay of DM and DT
(50-8000 ng/ml) were less than 10% and 5% respectively. The least quantifiable
quantity was 20 ng/ml for both DM and DT. In samples where DM peaks were
undetectable, concentration of DM was taken as the minimum quantifiable quantity
(20 ng/ml) for calculation.
Data analysis:

Phenotype was assigned with the metabolic ratio (MR) calculated as follows:

\[ MR = \frac{0-8h \text{ urinary output of unchanged dextromethorphan}}{0-8h \text{ urinary output of dextrophan}} \]

Analysis of interindividual variations in the metabolism of dextromethorphan was expressed by computing a histogram with \( \log_{10} MR \) on the X-axis and the number of subjects on the Y-axis. The Kolmogorov-Smirnov test was used to test the normality of the MR distribution. The antimode was considered as MR of 0.3\(^73\). The probit plot\(^71\) and normal test variable (NTV)\(^72\) were also computed to determine the mode of population distribution.

The probit analysis was performed on the \( \log_{10} \) of the MR. Probit value is determined for cumulative percentage of rank. To ensure that all the values are positive, +5 is added to the probit values. Probit plot is plotted using \( \log_{10} \) MR in the X axis and probit value in the Y axis. Inflection in the probit plot suggested the bimodality in the distribution of MR\(^71\).
**NTV plot**

The normal test variable plots for total study population and for individual states were made using the following procedure\(^7^2\):

- MR arranged in the ascending order.
- Converted the MR into their logarithmic form \(L\).
- The median (MD) and standard deviation (SD) of the logarithmic concentrations were calculated.
- The standardized form of each logarithmic concentration \(X\) is calculated using the formula:

\[
X = \frac{(L - MD)}{SD}
\]

- Cumulative number \(n\) is given for the MR.
- The cumulative frequency is \(F\) is calculated by dividing \(n\) by the total number \(N\) i.e. \(F = n/N\) (the value of \(F\) is in between 0 and 1)
- The secondary measure Z value is calculated using the formulae:

\[
Z = F - (1 - F) e^{1.6X}
\]

- NTV is calculated from the Z value

\[
NTV = -Z \quad \text{if } F \leq 0.5
\]
\[
NTV = \frac{Z}{e^{1.6X}} \quad \text{if } F \geq 0.5
\]

- The graph is plotted using \(F\) values in the X axis and NTV in the Y axis.
- Strong negative values in the NTV indicated the bimodality in the distribution.
Hardy Weinberg Law was used to compute the mutant and wild type allele frequencies of *CYP2D6* in South Indian population. The law states that the relative proportion of the genotypes would remain constant from one generation to another, and would occur in the proportion $p^2:2pq:q^2$. Here 'p' is the frequency of the wild type alleles and 'q' is the frequency of the mutant alleles.

The ratio of number of PM subjects ($n$) to the total number of subjects ($N$) is $q^2$. i.e. $q^2 = n/N$. Since $p + q = 1$, the $p$ can be calculated as $1-q$. The frequency of heterozygotes is calculated as $2pq$.

The influence of various factors like age, sex, food, body mass index, alcohol use, tobacco use and various allergies on the metabolic ratio was also analysed. Analysis of variants (ANOVA) with Bonferroni post-test was used to analyse the parameters between the four different states and three different religions. Spearman nonparametric correlation test was used to analyse the effect of age and body mass index on MR. The MR and BMI of male and female subjects compared using unpaired Student's t-test. All other comparisons were by Mann-Whitney test and $X^2$ test. $P < 0.05$ was considered as significant.

All the statistic analysis have been carried out using the GraphPad InStat version 3.00 for Windows 95 (GraphPad Software, San Diego California USA).