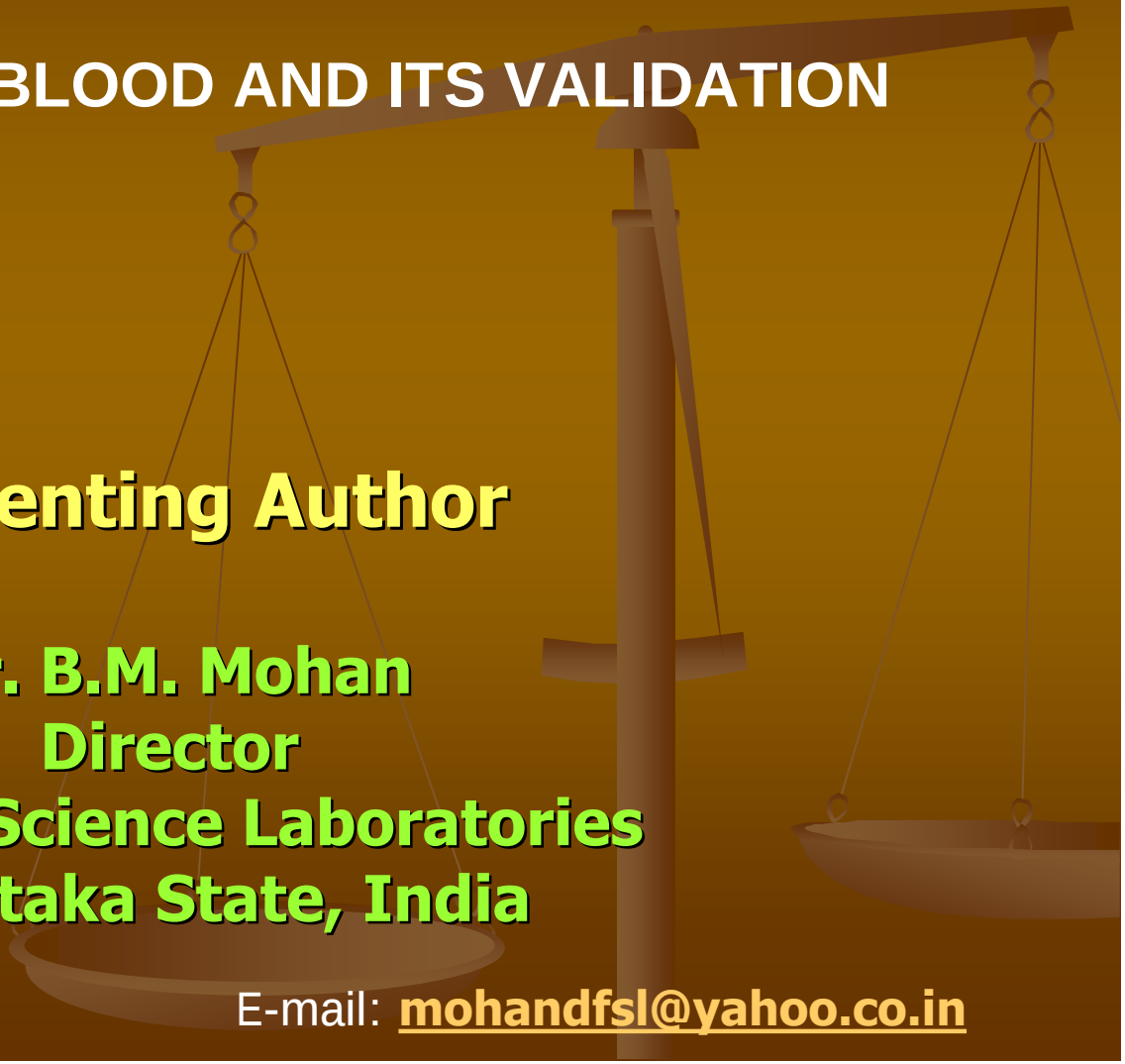
# A SIMPLE, ACCURATE AND RAPID HPTLC METHOD FOR THE DETERMINATION OF THEOPHYLLINE IN POST MORTEM BLOOD AND ITS VALIDATION

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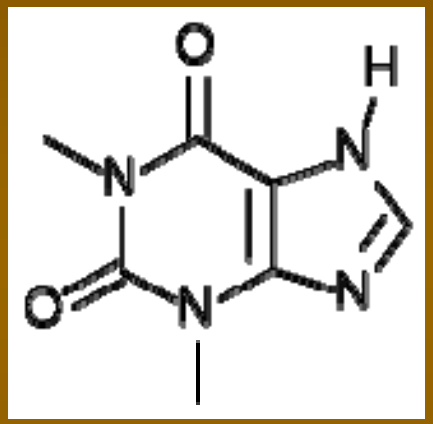
# Theophylline

- Theophylline (1, 3-dimethylxanthine) is used both in the prophylaxis of chronic asthma and chronic obstructive pulmonary disease (COPD), and to treat emergencies in acute severe asthma.




**($C_7H_8N_4O_2$)**
**1,3-dimethyl-7H-purine-2,6-dione**

- Theophylline works as a bronchodilator by the relaxation of bronchial smooth muscle.
- High incidences of effects of immunomodulations are reported even at relatively low plasma concentrations (05-15 µg/mL).
- At high concentrations, convulsions and cardiac arrhythmias occur.

- In chronic overdose, the severity of the overdose is more strongly correlated with a patient's age than with the theophylline concentration.
- Patients older than 60 years have the greatest risk for mortality and they are most likely to have seizures. These neurologic events often occur at lower theophylline concentration than those in acute overdose.
- This reinforces the need of not only monitoring plasma concentrations of theophylline, but also to examine whether the probable time of death in cases of fatalities due to excessive dosages could be evolved by quantifying the residual levels of theophylline or its metabolites from autopsied samples.

# Mechanism of action

Increases the intracellular concentrations of cAMP and cGMP by inhibiting phosphodiesterase; this causes the smoothening of bronchial muscle and allow the pulmonary blood vessels to relax .

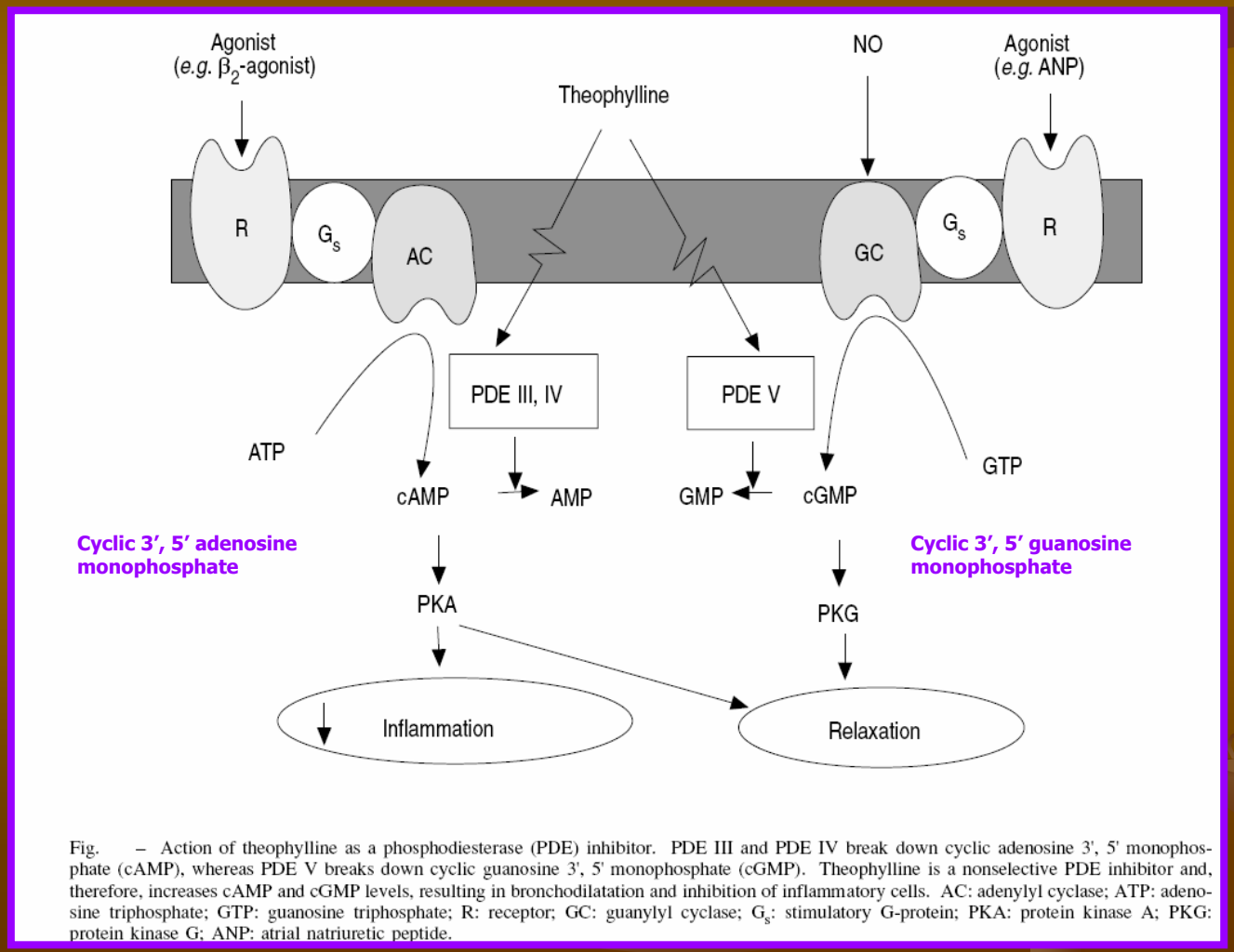


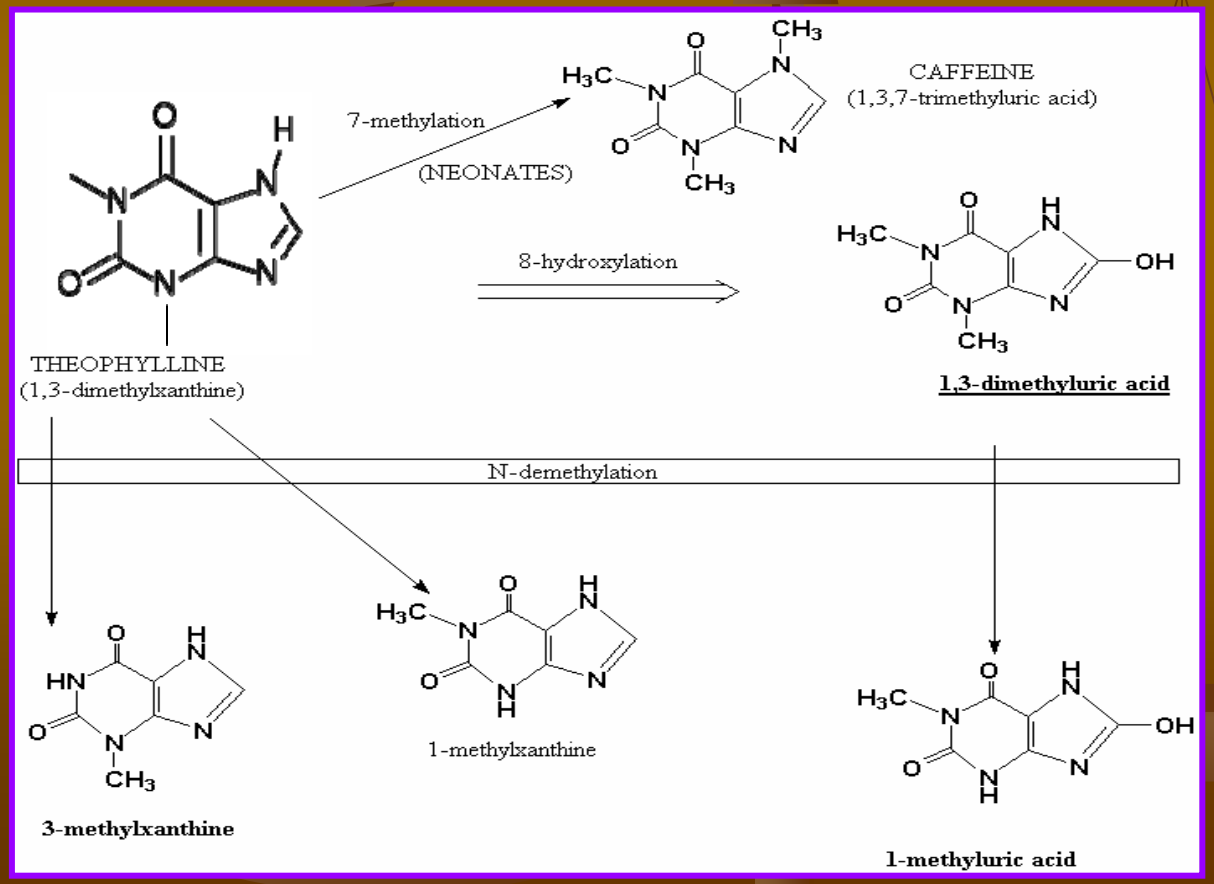

Fig. – Action of theophylline as a phosphodiesterase (PDE) inhibitor. PDE III and PDE IV break down cyclic adenosine 3', 5' monophosphate (cAMP), whereas PDE V breaks down cyclic guanosine 3', 5' monophosphate (cGMP). Theophylline is a nonselective PDE inhibitor and, therefore, increases cAMP and cGMP levels, resulting in bronchodilatation and inhibition of inflammatory cells. AC: adenylyl cyclase; ATP: adenosine triphosphate; GTP: guanosine triphosphate; R: receptor; GC: guanylyl cyclase; $G_s$: stimulatory G-protein; PKA: protein kinase A; PKG: protein kinase G; ANP: atrial natriuretic peptide.

# Metabolism

Theophylline is mainly metabolized in liver to 1, 3-dimethyluric acid, 3-methylxanthine, and 1-methylxanthine (1MX) mainly by cytochrome $P_{450}$ system, and 1MX is rapidly converted to 1-methyluric acid (1MU) by xanthine oxidase.

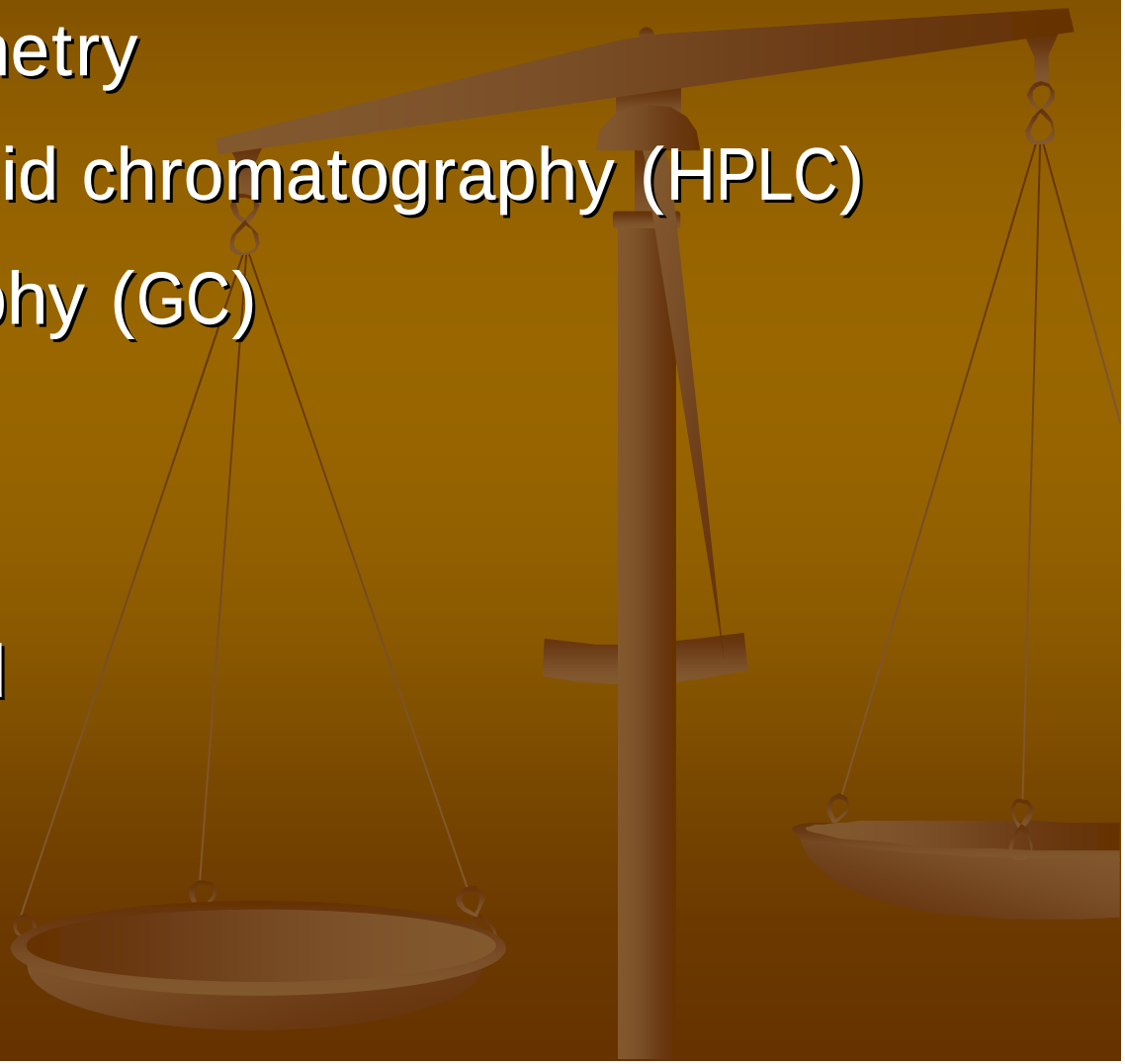
Metabolic pathway

- 13% of the dose excretes as unchanged.
- 15% as 3-methyl xanthine
- 35-50% as 1,3-dimethyl uric acid
- 20% as 1-methyl uric acid



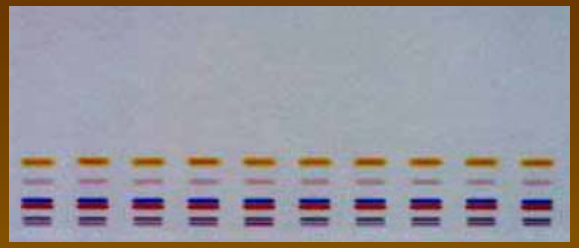
## Reported analytical methods for the quantitation of theophylline in biological fluids

- UV spectrophotometry
- High-pressure liquid chromatography (HPLC)
- Gas chromatography (GC)
- GC/MS
- LC/MS
- Immunoassay and
- Electrophoresis



- It is reported that the presence of several weakly acidic drugs cause false high and low values in spectrophotometric determination.
- The GC and HPLC methods require complex extraction procedures and some times derivatization.
- The other methods suffer from the disadvantages of being time consuming and require special procedures.
- None of the above analytical methods have potentials for their use in the analysis, from the post mortem blood.

# Why HPTLC ?



- Several samples can be run simultaneously.
- Separation of ten or twenty samples takes the same time as the separation of one sample using small quantities of solvents.
- This reduces the time and cost of analysis and decreases the possibility of environment pollution.
- Less sample clean-up is often required because plates are not reused. Every sample is analysed on a fresh layer without sample carryover or cross-contamination.
- Ability to generate a unique calibration curve using standards developed under the same conditions as samples on each plate (in-system calibration) leads to statistical improvement in data handling and better analytical precision and accuracy.
- Eliminates the need for an internal standard for most analysis.

# Extraction

- Post mortem blood (PMB) preserved in sodium fluoride was used.
- Direct extraction of theophylline from the PMB blood using chloroform:isopropanol (8:2) mixture resulted in the formation of emulsion and the separation of organic phase was tend to be time consuming.
- Alternatively, PMB was hydrolysed with $H_2SO_4$, followed by protein precipitation with sodium tungstate and the supernatant layer was separated by centrifugation. The pH of the supernatant was basified using ammonia.

# Extraction (continued…)

2 mL aliquot each of post mortem blood, blood calibrators, control samples

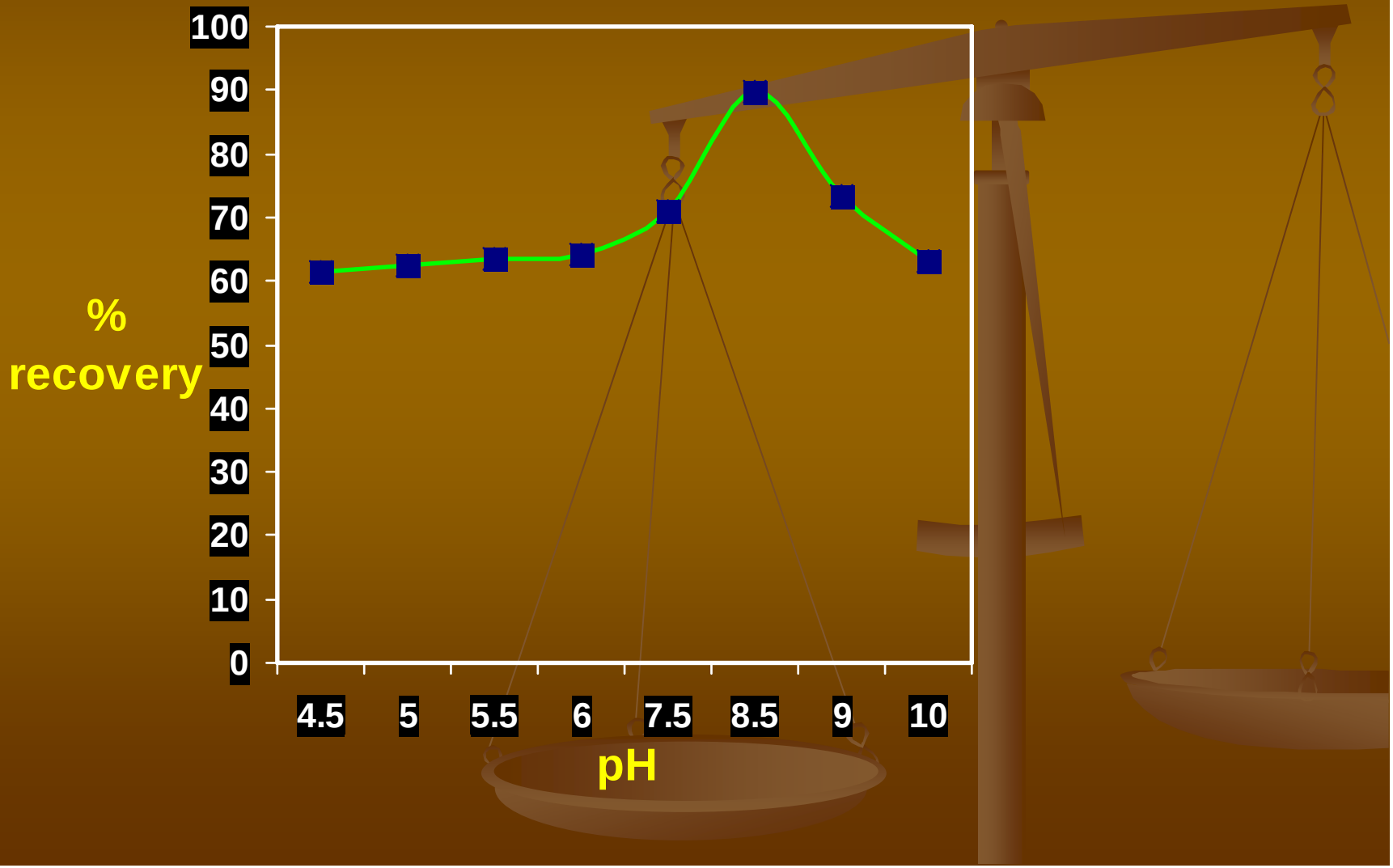
1 mL each of 10% Sod.tungstate and 1N $H_2SO_4$

Vortex mixed for 1 min and centrifuged at 3000 rpm for 10 min

Supernatant adjusted to pH 8.5 with 25% ammonia

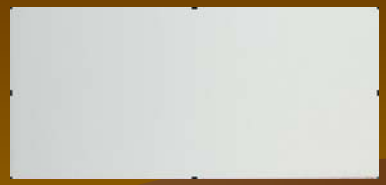
Vortex mixed for 02 min with 05 mL of chloroform-isopropanol (8:2) mixture. centrifugation for 10 min at 3000 rpm, the organic layer was evaporated to dryness and reconstituted with 0.5 mL methanol.

# Effect of pH on the extraction recoveries of theophylline from postmortem blood



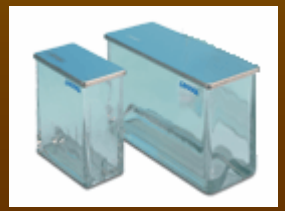
# Chromatography

**Performed on 20 cm × 10 cm aluminium-backed silica gel 60 $F_{254}$ HPTLC plates**

**Samples and standard solutions applied by means of a Camag Linomat 5 automatic sample applicator**

**Plates were developed in a glass twin-trough chamber, with chloroform-methanol (9:1 v/v), as mobile phase. The development distance was 8 cm from the lower edge of the plate and migration time 15 min.**



## Documentation

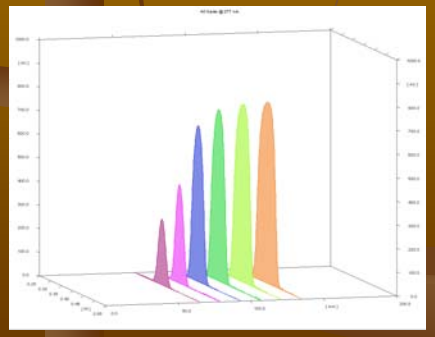
**Photo documentation at 254 nm and 366 nm using Camag Reprostar 3 digital documentation system.**

254 nm

366 nm

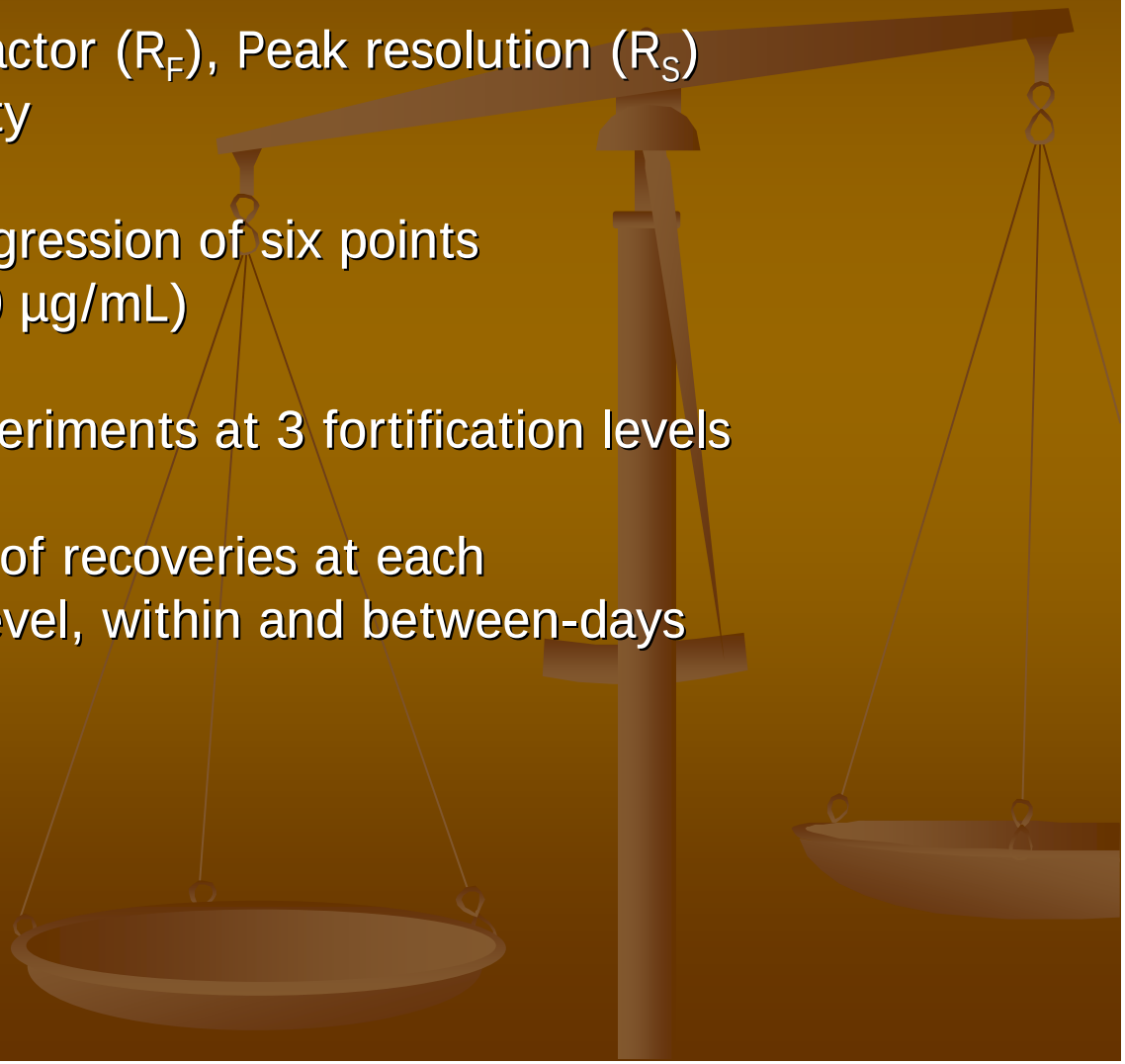
## Densitometry

***In situ*** **densitometry performed with a Camag TLC Scanner 3 equipped with CATS 3 software, in absorption mode at λ = 277 nm**

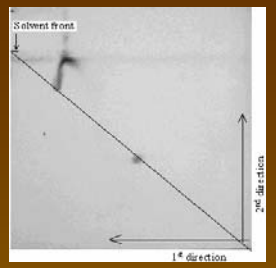


# Validation

- Stability : Analyte before and during chromatography
- Specificity : Retardation factor ($R_F$), Peak resolution ($R_S$) and Peak purity
- Linearity : Polynomial regression of six points (range 0.5- 20 µg/mL)
- Accuracy : Recovery experiments at 3 fortification levels
- Precision : Repeatability of recoveries at each fortification level, within and between-days
- Detection limit : At S/N = 3



# Stability




**No decomposition of theophylline was observed during chromatogram development.**

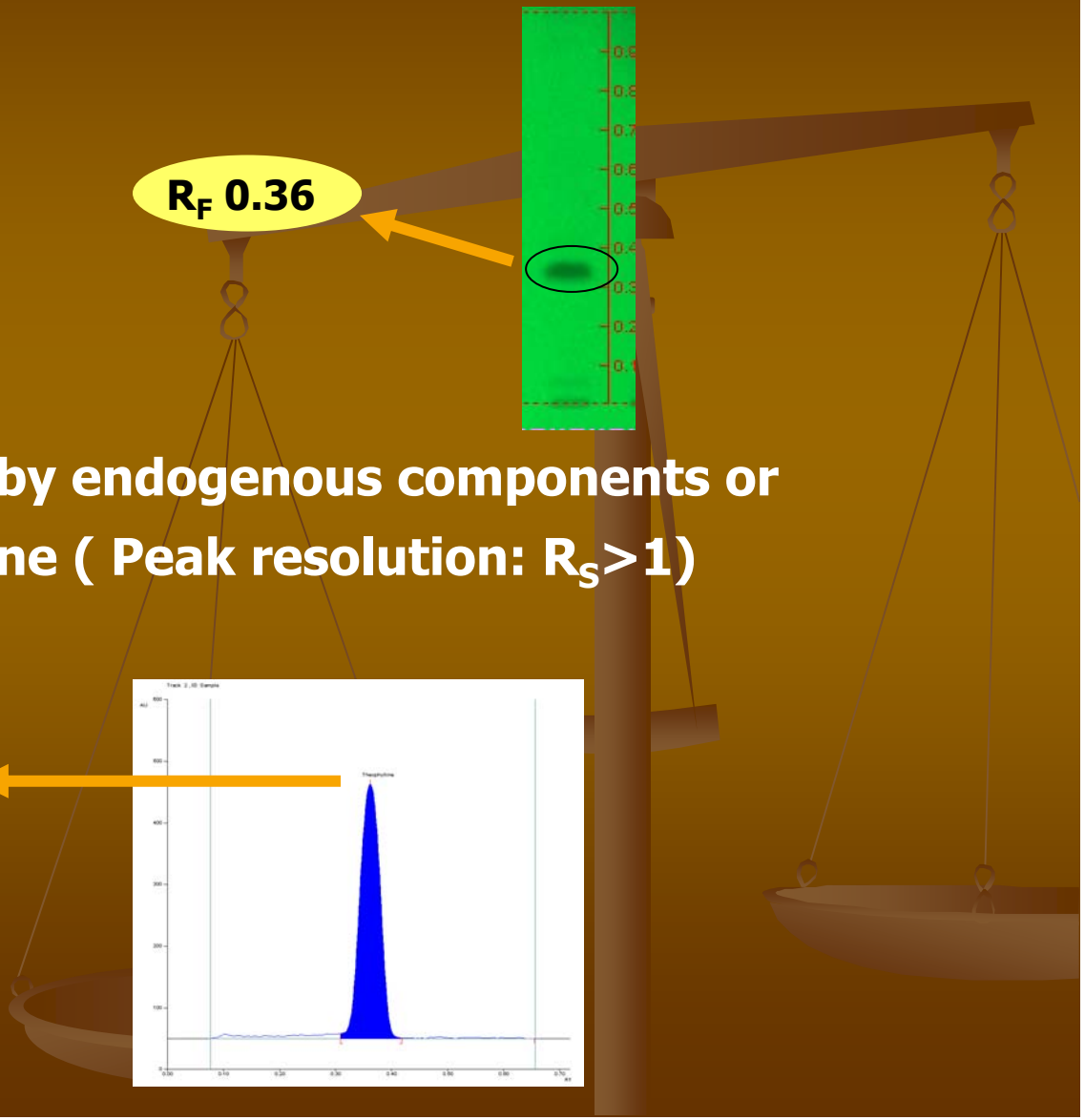
**The standard theophylline solutions found to be stable at room temperature in the solvent (methanol).**

| Chromatographic conditions | $R_F$ (RSD† %) (n=3) | RSD (%) of Peak area (n=3) |
|---|---|---|
| Sample on the plate after 3 h | 0.363(1.65) | 0.2 |
| Sample applied fresh | 0.357 (1.68) | 0.32 |
| Sample in the solution set-aside for 3 h | 0.357 (1.68) | 0.37 |
| Sample applied fresh | 0.360 (2.78) | 0.52 |
| Mean $R_F$<br>RSD % | 0.36<br>0.83% | |

† Relative standard deviation.

# Specificity

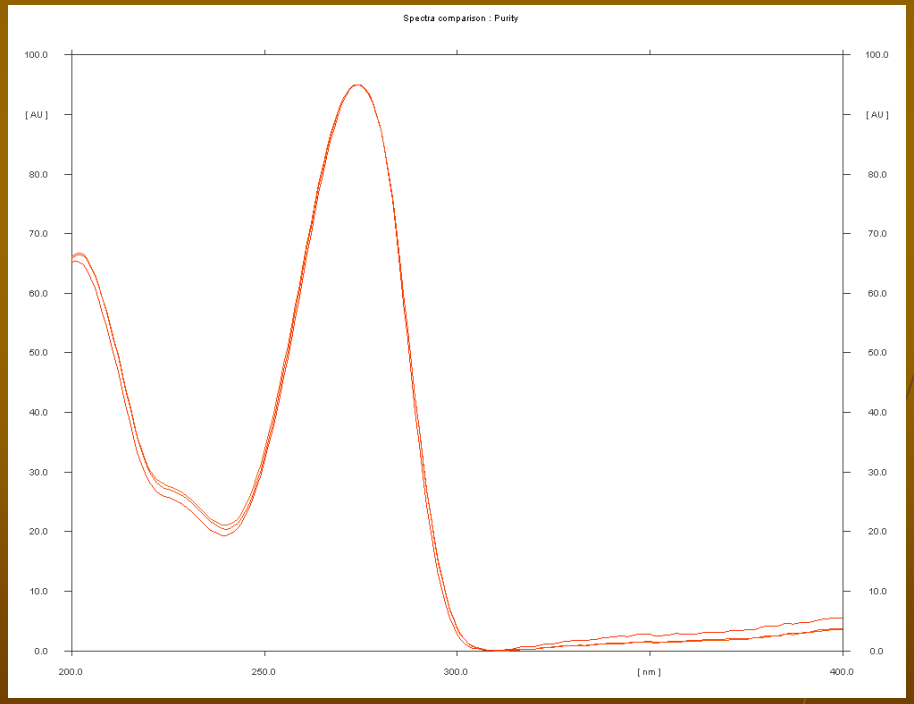
$R_F$ for theophylline; mobile phase: chloroform:methanol (9:1), is 0.36 ±0.01 (n=9), RSD= 0.83%



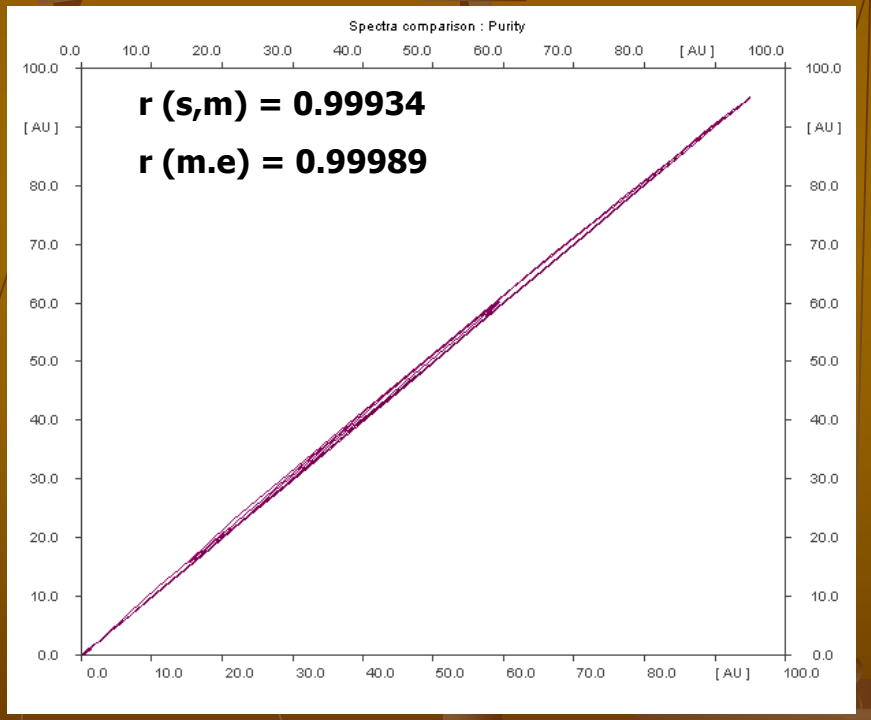

$R_F$ values are unaffected by endogenous components or metabolites of theophylline ( Peak resolution: $R_S$>1)

# Peak purity

**Peak purity in the PMB extract was reproducible when tested by correlating spectra acquired at the start (S), apex (A), and end (E) positions of the peak**




Center and slopes spectra

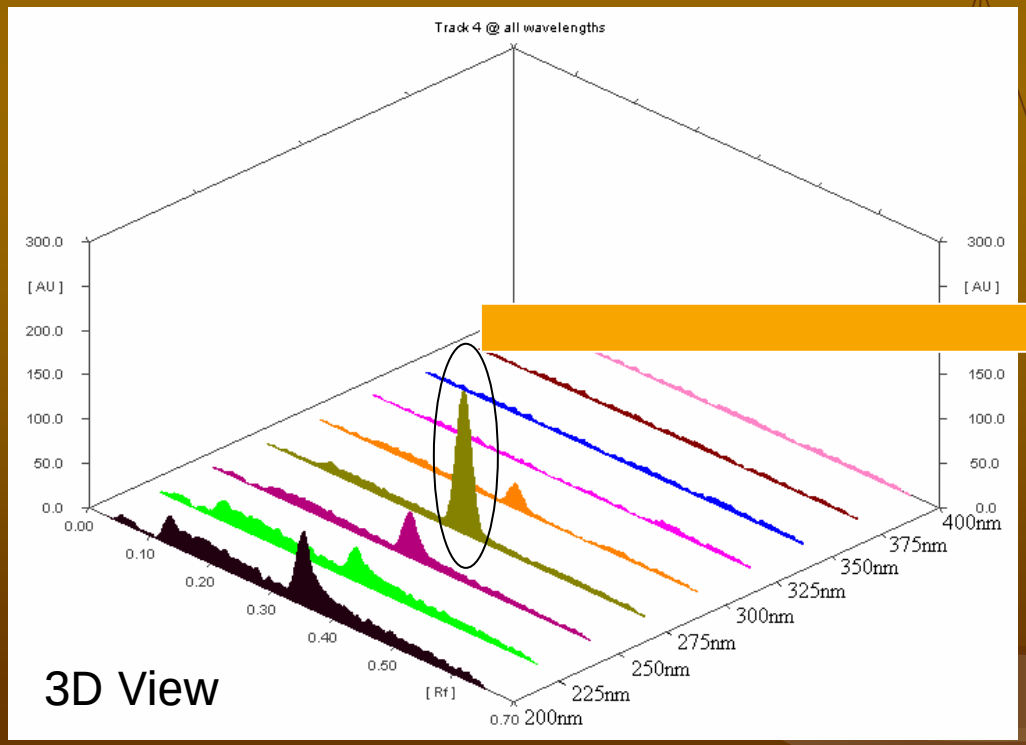


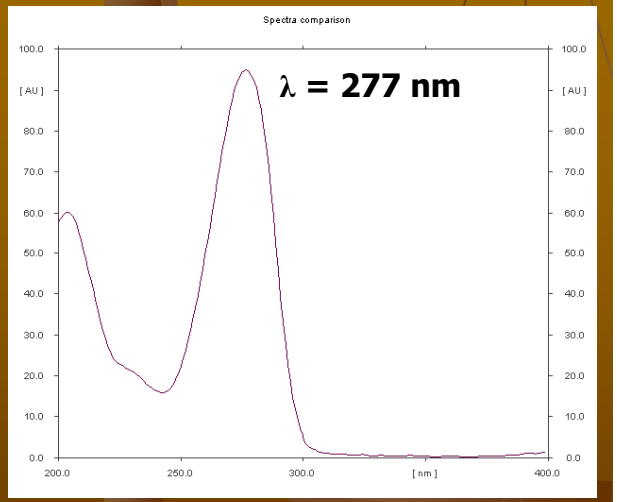

Peak-purity correlation display

# Maximum wavelength

## Optimization of wavelength of maximum absorption (detection wavelength for theophylline in the UV region)

**Multiwavelength scan (200 to 400 nm)**

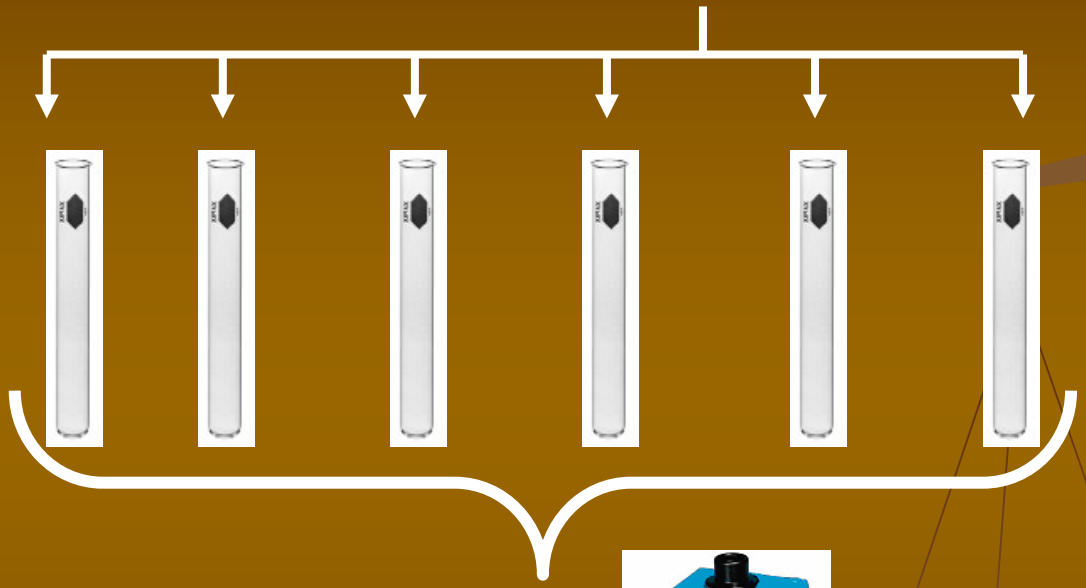


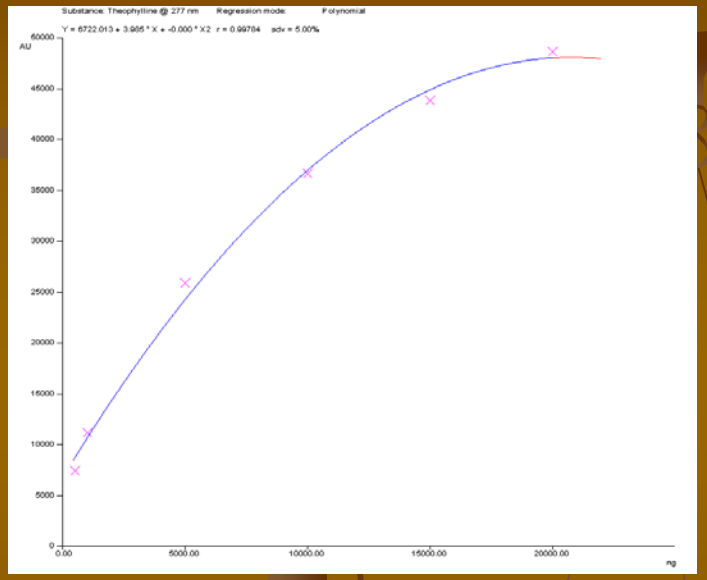




Absorption spectrum

# Linearity

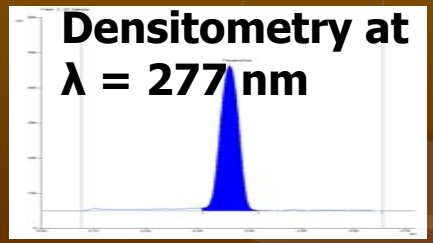
**Calibration range: 0.5 to 20 µg/mL PMB**





**Extraction**

**Chromatography**




**Regression mode: Polynomial**
**r = 0.998**
**sdv = 5%**

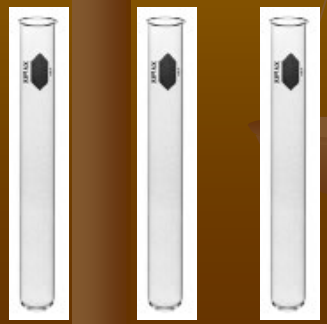
# Accuracy

**Theophylline standard solutions**

**0.5µg** **5µg** **10µg**

**Evaporated to dryness**

**Blood**



**Extraction**

**% Recovery**

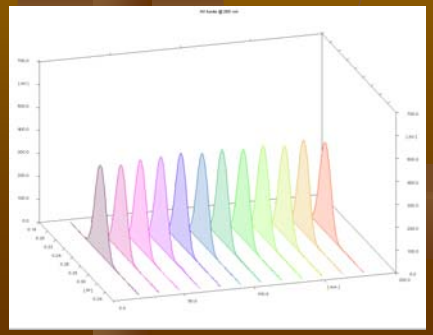
$$\frac{\text{Quantified value}}{\text{Spiked value}}$$

## Statistical recovery and precision data for theophylline, in spiked post mortem blood.

| Spiked Amount (μg/mL) | Relative Recovery (Mean), (%) | Precision (RSD %†) | |
|---|---|---|---|
| | | Intra-day repeatability | Inter-day repeatability |
| 0.5 | 87.85 ± 0.65 | 0.8 | 1.3 |
| 5.0 | 90.16 ± 0.44 | 0.49 | 0.66 |
| 10 | 91.77 ± 0.57 | 0.62 | 0.53 |
| Average recovery | 89.93 ± 1.97 | | |
| RSD% | 2.19 | | |

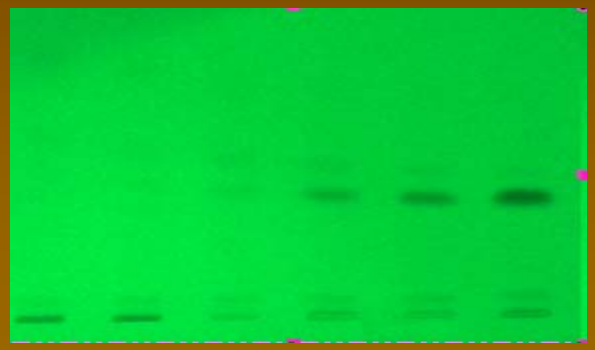
**Instrument precision**

No changes in → $R_F$

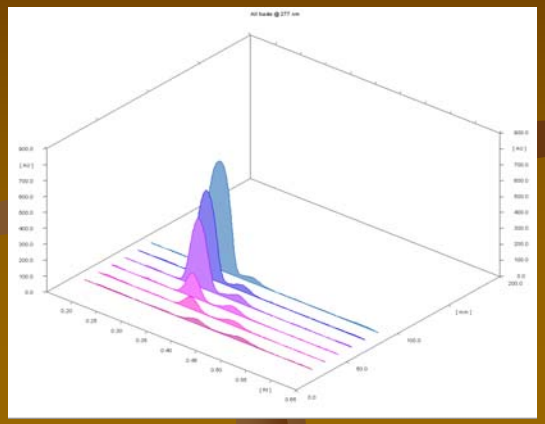


Peak area

# Detection Limit



Decreasing quantities



Chromatogram

**LOD = 0.5µg/mL**

**S/N = 3**

# Application to medico-legal case

**History:**

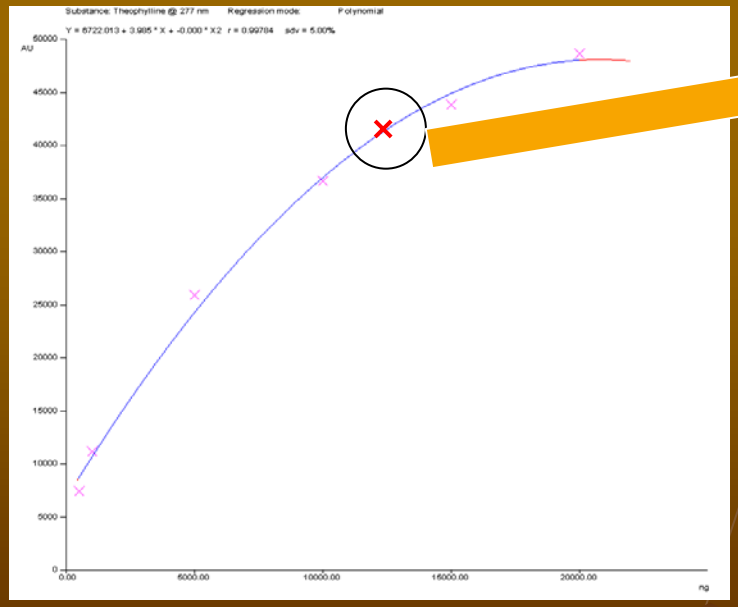
- Deceased was a 75-year-old female with a history of chronic asthma.
- Due to severity of asthma, she was reported to have consumed excess tablets and admitted to hospital.
- She was declared dead after the treatment for 4 days.

**Autopsied samples:**

- Liver, kidney, blood and stomach specimens were sent for toxicological analysis.

## Quantification

**The concentration of theophylline estimated in PMB was 13 μg/mL, which lies within the therapeutic range.**




**13 μg/mL**

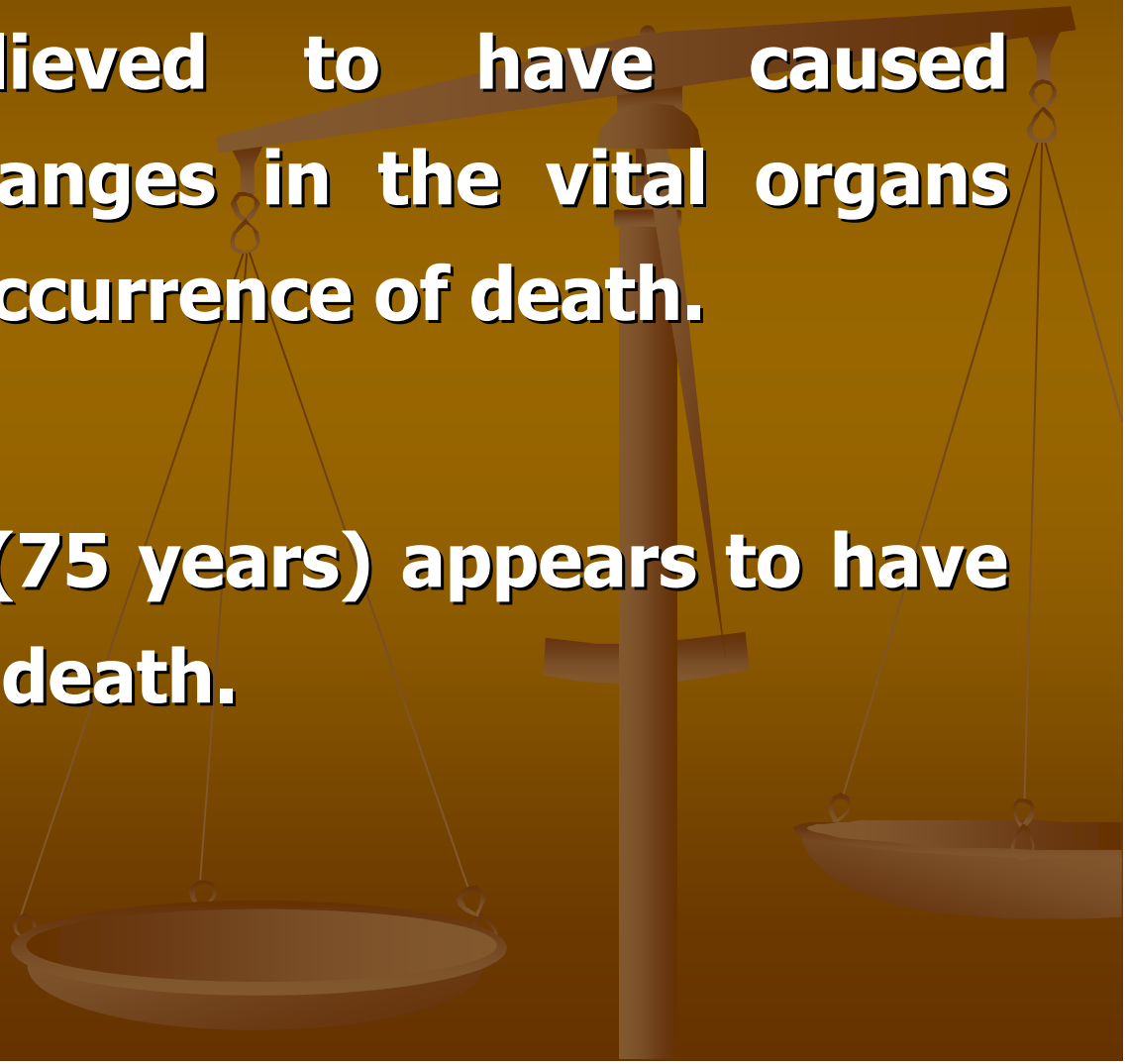
**Therapeutic range**

**05-15 μg/mL**

## Implications

- The low concentration of theophylline found, is believed to have caused pathological changes in the vital organs leading to the occurrence of death.

- The age factor (75 years) appears to have accelerated the death.



## Conclusion:

- The extraction procedure developed was simple yielding good recoveries with reproducibilities.
- The proposed HPTLC method has the advantages of simplicity, speed, accuracy and reproducibility and does not require extensive clean-up procedures and with no sample carryover or cross-contamination.
- The analytical data for theophylline in post mortem blood is of significant help in evolving causes of death in case of theophylline poisoning.

# Research team

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Thank you
Thank you