

# DEVELOPMENT OF A HIGH SENSITIVITY IMMUNOASSAY FOR THE MEASUREMENT OF TRAMADOL AND ITS MAJOR METABOLITES.

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## INTRODUCTION

Tramadol is a centrally acting analgesic for the treatment of moderate to severe pain. It has been shown to have a relatively weak affinity for the  $\mu$ -opioid receptor compared to morphine, and its potential for abuse is considered low. Consequently, there is unrestricted access to the drug throughout Europe and it is unscheduled in the USA. However, individual reports and studies have shown that tramadol can provoke addictive and abusive behaviour (1,2). N-demethyltramadol and O-demethyltramadol are the major metabolites of tramadol. Demethylation is catalysed in the liver by isoforms of cytochrome P-450. Some individuals lack the CYP2D6 enzyme, necessary for O-demethylation of tramadol, and consequently produce low levels of this compound (3,4). Tramadol and its two major metabolites are excreted in the urine.

This study reports the initial development of a high sensitivity, one step, nonradioactive immunoassay for the measurement of tramadol and its metabolites in biological fluids without sample pre-treatment.

## MATERIALS AND METHODS

The competitive immunoassay employed was performed using in-house generated target-specific polyclonal antibodies and horseradish peroxidase (HRP) labelled conjugates. A 96 well microtiter plate was precoated with the specific capture antibodies. Competition between free tramadol and tramadol metabolites present in the calibrator/sample and HRP-labelled conjugates for antibody binding sites, after incubation of 1 hour at 25°C, was measured by reading the absorbance at 450 nm. The absorbance was inversely proportional to the concentration of the analytes.

### • Assay evaluation parameters

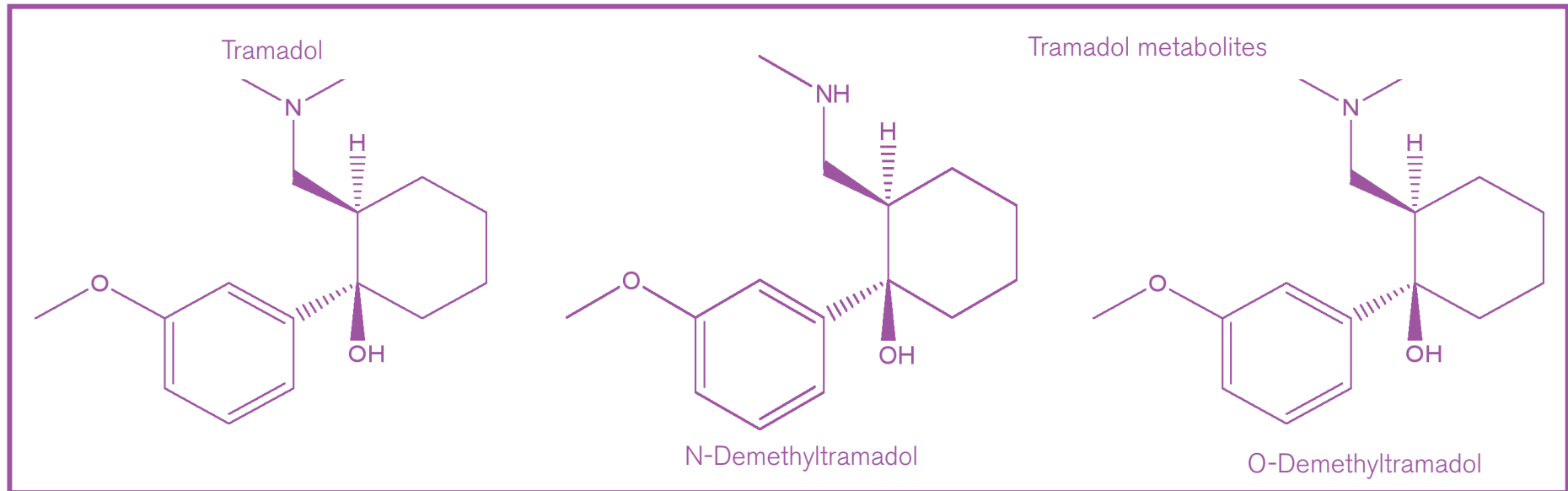
Calibration curves : The calibration curves were generated with each of the analytes as standards in the competitive immunoassay.  $B/B_0$  values were calculated, where B is the absorbance measured at 450nm for x ng/ml of the analyte and  $B_0$  is the absorbance measured at 450nm in the absence of analyte.

IC50: The IC50 for each analyte was calculated by taking 50% of the optical density (OD) from the zero calibrator and reading this OD value from the x-axis (concentration in ng/ml) of the respective calibration curve. This concentration corresponded to the inhibitory concentration that produced 50% inhibition.

Cross-reactivity: The cross-reactivity was calculated as follows:

$$\% \text{ Cross-reactivity (\%CR)} = [\text{IC50 (Tramadol)}/\text{IC50 (Cross-reactant)}] \times 100$$

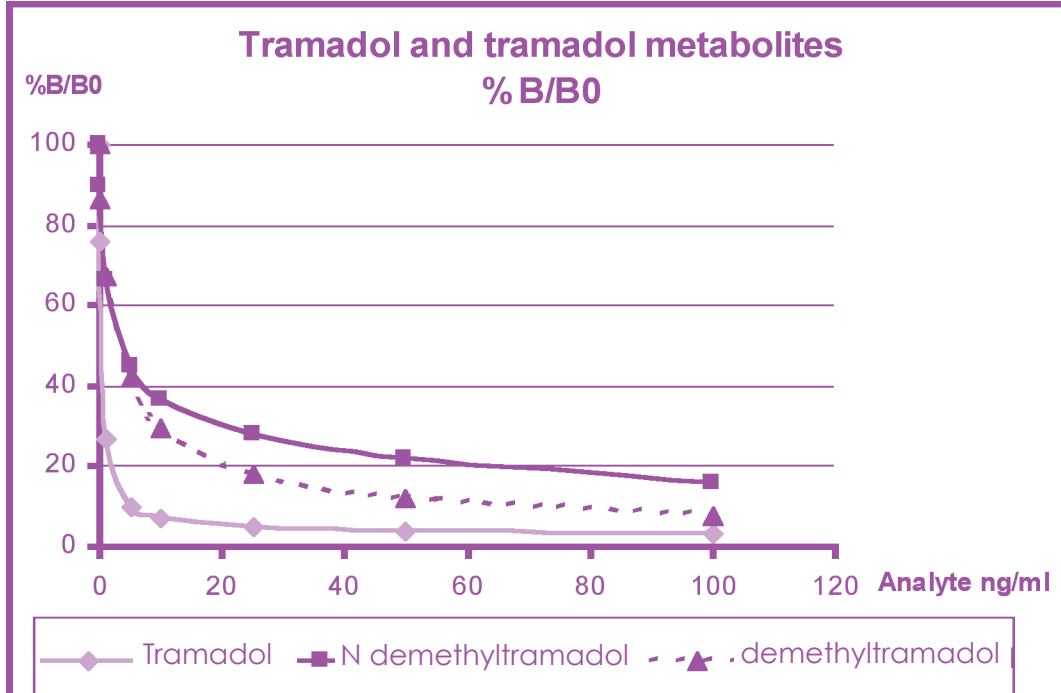
Intra-assay precision: Five concentration levels of the analytes were used and the intra-assay precision was determined from the results of three replicates of each level within the same run. The precision was expressed as %CV.



## RESULTS

Performance parameters of the initial evaluation of the competitive immunoassay:

Calibration curves for tramadol and tramadol metabolites in the competitive immunoassay.



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Low levels of this metabolite in CYP2D6 deficient individuals

### Sensitivity

Analyte	IC50 (ng/ml)
Tramadol	0.32
N - demethyltramadol	3.43
O - demethyltramadol	3.01

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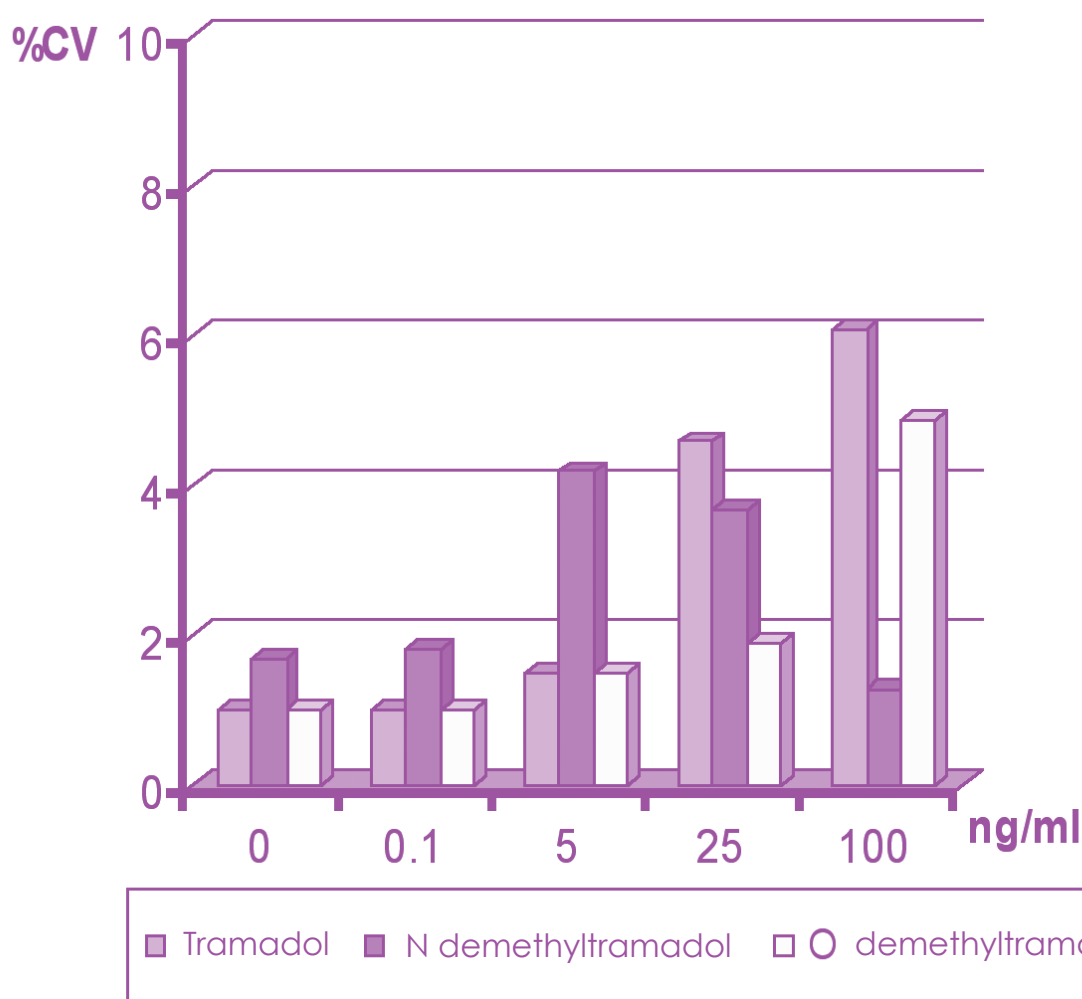
### Specificity

Analyte	% Cross Reactivity
Tramadol	100
N - demethyltramadol	9.2
O - demethyltramadol	10.5

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Comparison of reported immunoassay sensitivity with sensitivity technical data from other commercial immunoassays:

	Reported Immunoassay	Commercial immunoassay 1	Commercial immunoassay 2
Analyte	IC50 (ng/ml)	IC50 (ng/ml)	IC50 (ng/ml)
Tramadol	0.32	5	200
N-demethyltramadol	3.43	83.3	>10000
O-demethyltramadol	3.01	18.5	500
Metabolite ratio	1: 1.1	1: 4.5	>1: 20



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## CONCLUSIONS

The initial evaluation in the development of this one step, nonradioactive immunoassay shows that tramadol and its major metabolites, N-demethyltramadol and O-demethyltramadol, are detected with high sensitivity and specificity at concentrations below 4 ng/ml. In this immunoassay the IC50 concentrations for the two metabolites are similar, which improves the detection levels when compared with current commercial assays. This immunoassay facilitates the detection of tramadol and both of its metabolites in a sample, which is significant in CYP2D6 deficient individuals who underproduce O-demethyltramadol.

### REFERENCES

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