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2 **DETERMINATION OF METHYLPHENIDATE IN PLASMA AND SALIVA BY LIQUID**  
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4 **CHROMATOGRAPHY/TANDEM MASS SPECTROMETRY**  
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## ABSTRACT

1  
2 Methylphenidate (MPH) is a phenethylamine derivative used in the treatment of attention-  
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4 deficit hyperactivity disorder (ADHD). In adults, clinical monitoring of MPH therapy is usually  
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6 performed by measuring plasma MPH concentrations. In children blood sampling is however  
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8 undesirable. Saliva may be an alternative matrix for monitoring MPH concentrations with the  
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10 advantage that it can be obtained non-invasively. Therefore, we developed an analytical  
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12 method for the quantification of MPH in both plasma and saliva.  
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15 We present the validation of a liquid chromatography - tandem mass spectrometric method  
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17 using a hydrophilic interaction liquid chromatography column (HILIC). In 100  $\mu\text{L}$  sample,  
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19 proteins were precipitated with 750  $\mu\text{L}$  acetonitrile/methanol 84/16 (v/v) containing d9-  
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21 methylphenidate as the internal standard. Standard curves were prepared over the MPH  
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23 concentration range of 0.5 – 100.0  $\mu\text{g}\cdot\text{L}^{-1}$ . The total analysis time was 45 seconds. Accuracy  
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25 and within- and between-run imprecision were in the range of 98-108% and less than 7.0 %,  
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27 respectively. Matrix effects were greater for plasma than saliva with 46% and 8% ionization  
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29 suppression. The matrix effects were adequately compensated by the use of deuterated  
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31 MPH as internal standard. MPH significantly degraded in plasma and saliva at room  
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33 temperature and 5°C. Samples were stable at -20°C for at least 4 weeks. The method was  
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35 successfully applied for the determination of MPH concentrations in plasma and saliva  
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37 samples from an adult healthy volunteer. Using protein precipitation and hydrophilic  
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39 interaction liquid chromatography coupled to tandem mass spectrometry, this method allows  
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41 fast, accurate and precise quantification of MPH in both plasma and saliva.  
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49 Keywords: methylphenidate, LC-MS/MS, saliva, plasma, HILIC.  
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## 1. INTRODUCTION

Methylphenidate (MPH) is a psychostimulant widely used in the treatment of attention-deficit hyperactivity disorder (ADHD). ADHD is a neurobehavioral problem mostly encountered in school-aged children at a prevalence of 5-10% of the general population<sup>[1,2]</sup>. MPH is a piperidine-derived molecule that contains two chiral centers and exists as four stereoisomers. The pharmacological activity resides entirely with the *dl-threo*-methylphenidate racemic (50:50) mixture<sup>[3]</sup>.

The major metabolic pathway of MPH is the hydrolysis of the methyl ester linkage by esterases to form ritalinic acid<sup>[4-5]</sup>. Minor metabolic pathways for both these compounds include parahydroxylation of the aromatic ring, oxidation to 6-oxo-derivatives and glucuronide formation<sup>[6-7]</sup> (Figure 1). Ritalinic acid and the other metabolites are pharmacologically inactive<sup>[8-9]</sup>.

There is a clinical need to perform therapeutic drug monitoring (TDM) in patients who are undergoing MPH therapy. MPH exhibits wide inter-individual variability in both pharmacokinetics and clinical response<sup>[10-11]</sup>. TDM can be applied when the patient remains unresponsive to therapy, exhibits unexpected adverse events or to check adherence. In adults, clinical monitoring of MPH therapy is usually performed by measuring plasma MPH concentrations. In children, monitoring of drug levels should be performed with minimal discomfort for the patient. Monitoring of MPH concentrations in saliva may therefore be an interesting non-invasive alternative to blood sampling.

Several methods have been developed for quantification of MPH in plasma, urine and hair, using high-performance liquid chromatography (HPLC) with ultraviolet detection<sup>[6,12]</sup>, capillary electrophoresis-mass spectrometry<sup>[13]</sup>, gas chromatography-mass spectrometry<sup>[8,9,14]</sup> and liquid chromatography–tandem mass spectrometry<sup>[7,15-17]</sup>. The determination of MPH concentrations by standard reversed-phase (RP) chromatography coupled to MS/MS detection is particularly challenging since retention times are generally very short due to the high hydrophilicity of the compound. This produces a significant loss in sensitivity due to the co-elution with matrix interference and the high percentage of water at

1 the chromatographic elution time. Recent research however has shown that for hydrophilic  
2 compounds the sensitivity, precision and accuracy of a quantitative analytical  
3 chromatographic method may be improved by using hydrophilic interaction liquid  
4 chromatography (HILIC) [18]. In addition, the use of HILIC has advantages in sample  
5 preparation when measuring polar compounds. Because of the high organic modifier  
6 content, usually acetonitril, used during chromatography, proteins can be precipitated using  
7 organic solvents without the loss of chromatographic integrity, as is often the case when  
8 used with polar compounds in combination with RP chromatography. A high organic modifier  
9 concentration is also ideal for compound ionization by electrospray ionization mass  
10 spectrometry (ESI-MS), resulting in higher sensitivity.

11 The aim of the present study was to develop a method to determine MPH concentrations in  
12 human plasma and saliva for potential use in therapeutic drug monitoring. We present the  
13 development and validation of an analytical method using HILIC chromatography coupled to  
14 tandem mass spectrometry. The stability of MPH in plasma and saliva was investigated at  
15 different temperatures. The applicability of the method was demonstrated with plasma and  
16 saliva data from one healthy adult volunteer obtained before and after intake of 10 mg  
17 immediate release (IR) MPH and 18 mg MPH - osmotic controlled-release oral delivery  
18 system (OROS) - on different occasions.

## 2. MATERIALS AND METHODS

### 2.1 Chemicals

19 Methylphenidate was purchased from Bufa (Uitgeest, Netherlands). As an internal standard  
20 (I.S.) a 1 mg/mL solution of deuterated methylphenidate HCl ( $d_9$ -MPH) in methanol was  
21 obtained from LGC-Standards (Teddington, United Kingdom) (Figure 2). Water was purified  
22 and deionized using an ELGA purelab Optron Q (Veolia Water; Saint Maurice, France). Drug

1 free, non sterile, K<sub>2</sub> EDTA human plasma was obtained from Equitech-Bio (Kerrville - TX,  
2 USA). OraFlx synthetic saliva was obtained from Dyna-Tek (Lenexa - KS, USA).  
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## 6 2.2 Instrumentation 7

8 The LC-MS setup comprised of a Thermo Scientific Surveyor LC (Waltham - MA, USA)  
9 system coupled to a Maylab Mistraswitch column oven (Spark Holland, Netherlands  
10 Emmen), and a Thermo Scientific TSQ Quantum Access MS system with an ESI source.  
11 The Xcalibur 2.0.7 SP1 (Thermo Scientific) software package was used for controlling the  
12 LC-MS system and for data processing.  
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## 22 2.3 LC-MS/MS conditions 23

24 Isocratic elution was applied using A: 2% formic acid in water (v/v) and B: acetonitril 100%. A  
25 was set at 10% and B at 90%. Analytical separation was accomplished on a SeQuant ZIC-  
26 HILIC column (Merck, Darmstadt, Germany) with a length of 50 mm, an internal diameter of  
27 2.1 mm and 5 μm particle size. The flow rate was 1.00 mL/min giving a total  
28 chromatographic run time of only 45 seconds. To minimize carry-over effects the LC  
29 injection system was washed with 20% formic acid in water (v/v) after every injection. The  
30 autosampler temperature was maintained at 10°C, the column oven at 30°C. The analytes  
31 were detected in positive ion mode using multiple reaction monitoring (MRM). The ion spray  
32 voltage was 5000 V and the ion transfer tube temperature was 250°C. Sheath and auxiliary  
33 gas pressure were 50 and 20 psi, respectively. Collision gas (argon) pressure was 2.0  
34 mTorr. MPH and d<sub>9</sub>-MPH were measured as [M+H]<sup>+</sup> using the mass transitions 234.1 →  
35 84.1 and 243.1 → 93.1 respectively. Tube lens voltage and collision energy were 90 and 21  
36 V, respectively. Dwell time was 300 ms for MPH and 50 ms for d<sub>9</sub>-MPH.  
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## 2.4 Analytical procedures

### 2.4.1 Preparation of stock solutions

A standard stock solution was prepared in water containing 1.0 mg/mL MPH. Two MPH stock solutions were prepared by dissolving 50  $\mu$ l and 500  $\mu$ L of the 1.0 mg/ml MPH solution in 10 ml water/methanol 1/1 (v/v); corresponding concentrations were 5.0 mg/L and 50.0 mg/L. The stock internal standard was prepared by diluting 5  $\mu$ l of the d<sub>9</sub>-MPH 1 mg/ml standard solution to a total volume of 500 ml with acetonitrile/methanol 84/16 (v/v). The final concentration of the internal standard was 10  $\mu$ g/L. All stock solutions were stored at 5°C until use.

### 2.4.2 Preparation of calibration standard and quality control solutions

Four MPH calibration standard solutions were prepared by diluting 10, 20, 50, and 100  $\mu$ l of the 5.0 mg/L stock solution in 10 ml of water/methanol 1/1 (v/v). The concentrations were 5.0, 10.0, 25.0, 50.0  $\mu$ g/L, respectively. Three MPH calibration standard solutions were prepared by diluting 20, 80 and 200  $\mu$ l of the 50.0 mg/L stock solution in 10 ml of water/methanol 1/1 (v/v). The concentrations were 100, 400 and 1000  $\mu$ g/L, respectively. Quality control (QC) solutions were prepared in a similar manner as the calibration standard solutions. The MPH concentrations of the QC solutions were 5.0, 100 and 1000  $\mu$ g/L. The calibration standard and QC solutions were stored at 5°C until use.

### 2.4.3 Sample preparation

Calibration standards and QC samples were prepared just prior to analysis. Calibration standard and QC solutions were shortly vortexed and a volume of 10  $\mu$ l was pipetted into a 1.8 ml vial. Subsequently, 100  $\mu$ L saliva or plasma, depending on the composition of the calibration line, was added and shortly vortexed. Final concentrations of the plasma and saliva calibration line were 0.5, 1.0, 2.5, 5.0, 10.0, 40.0 and 100.0  $\mu$ g/L and final concentrations of the quality controls were 0.5 (QC1 (LLOQ)), 10.0 (QC2) and 100.0  $\mu$ g/L (QC3). Patient plasma and saliva samples were thawed and shortly vortexed and 100  $\mu$ l of

1 each sample was pipetted into a 1.8 ml vial. Subsequently, 10 µl of water/methanol 1/1 (v/v)  
2 was added and shortly vortexed. In all samples proteins were precipitated by adding 750 µL  
3 of the internal standard solution. After vortexing for 1 minute, samples were stored at -20°C  
4 for 30 minutes, vortexed again and centrifuged for 5 minutes at 4800g. Two microliter of the  
5 supernatant was injected.  
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#### 10 11 12 *2.4.4 Quantification*

13 MS response was expressed as integrated area of the chromatographic peak. For  
14 calibration, the concentration of prepared calibration standards was the known variable (x),  
15 the ratio of analyte MS response divided by internal standard MS response per calibration  
16 level was the unknown variable (y). Patient samples were back-calculated using the  
17 calibration line by their respective ratio of analyte / internal standard MS response.  
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### 28 *2.5 Method validation*

#### 29 *2.5.1 Selectivity*

30 One lot of blank, commercially acquired saliva and plasma, together with saliva and plasma  
31 samples from five different patients, not receiving MPH, were tested for interferences.  
32 Proteins were precipitated using acetonitrile/methanol 84/16 (v/v) without I.S. The data of the  
33 chromatograms were processed and the integrated response should not exceed 10% of the  
34 average integrated response of the LLOQ of MPH and 1% of the integrated response of d<sub>9</sub>-  
35 MPH.  
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#### 49 *2.5.2 Calibration*

50 A total of six calibration lines, consisting of seven different concentrations, were prepared in  
51 commercially acquired saliva and plasma and measured during six runs. Calibration curves  
52 were obtained by fitting the peak area ratios to a weighted (1/x) least squares regression  
53 model.  
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### 2.5.3 Accuracy and imprecision

The accuracy and imprecision of the method were determined for the QC samples during six consecutive runs. In the first run all QC concentration levels were analyzed in six fold (within-run imprecision); during the following five runs a single sample of each level was analyzed (between-run imprecision). Mean accuracy and within-run imprecision (coefficient of variation) were calculated from the results (n=6) of the first run. Between-run imprecision was calculated from the results (n=6) of the first sample of the first run and the samples of run two through six. According to the US Food and Drug Administration guideline for bio-analytical method validation the mean accuracy should be within 85-115% and the within-run and between-run imprecision should be less than 15% <sup>[19]</sup>. Furthermore, the limit of quantification of the assay was defined as the lowest concentration of MPH that could be detected with a mean accuracy within 80-120% and within-run and between-run imprecision not exceeding 20% of the coefficient of variation <sup>[19]</sup>.

Since plasma and saliva may be diluted to obtain concentrations in the calibration range, the accuracy of diluted samples was determined as well. Plasma and saliva samples were prepared with concentrations of 100 µg/L (QC3) and 1000 µg/L. All samples were diluted ten times with commercially acquired saliva and plasma (10 µL sample + 90 µL plasma/saliva) in six fold and the accuracy was determined. Mean accuracy of the diluted samples should be within 85%-115% and imprecision should be less than 15%.

### 2.5.4 Process efficiency and matrix effects

Plasma, saliva and solvent components in the ionization chamber may cause batch specific ion suppression or enhancement, leading to inter-patient and intra-patient signal variability<sup>[20-21]</sup>. Assay recovery and matrix effects were quantified for both plasma and saliva using the strategies proposed by Matuszewski *et al* <sup>[22]</sup>. In short, chromatograms were obtained from plasma and saliva samples that were spiked pre-precipitation, plasma and saliva samples spiked post-precipitation and spiked aqueous solutions. In total, six batches of plasma and saliva were spiked in duplicate; the MPH and d<sub>9</sub>-MPH concentrations were 10 µg/L.

1 Recovery (RE) was defined as the relative signal of samples spiked post-precipitation versus  
2 pre-precipitation. Matrix effects (ME) were similarly defined as the relative signal of post-  
3 precipitation spiked plasma and saliva samples versus spiked aqueous samples. A value of  
4 100% for ME indicated that signals in plasma/saliva samples and aqueous samples phase  
5 were similar. A ME value greater than 100% indicated ionization enhancement, whereas a  
6 value less than 100% indicates ionization suppression.  
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10 Process efficiency (PE) was defined as the product of RE and ME, i.e. the overall signal of  
11 spiked plasma and saliva versus an aqueous standard solution. Average values and  
12 coefficients of variation of RE, ME and PE were calculated over the six plasma and saliva  
13 batches.  
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#### 24 *2.5.5 Stability*

25 The stability of MPH in saliva and plasma QC1 (LLOQ) and QC3 samples was determined  
26 for several storage conditions. The freeze-thaw stability in plasma and saliva was  
27 determined by comparing freshly prepared samples with samples that underwent three  
28 freeze-thaw cycles (24 hours at -80°C). The MPH concentration of plasma and saliva  
29 samples stored at -20°C and -80°C was determined weekly and compared with freshly  
30 prepared samples. The stability of MPH in plasma and saliva at 5°C was assessed after 2, 5  
31 and 7 days of storage. The time course of MPH degradation in plasma and saliva was  
32 studied at room temperature by determination of the MPH concentration at the start of the  
33 experiment and 1, 4, 8, 21.5, 24 and 48 hours after the start. The esterase mediated decay  
34 of MPH in plasma and saliva was described by a first-order process. Data were log-  
35 transformed and rate constants were obtained by linear regression. Half life was calculated  
36 by dividing 0.693 by the rate constant.  
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53 The MPH concentration of processed samples stored in the autosampler (10°C) was  
54 determined after 24 hours and compared with the initial concentration. The analyte was  
55 considered stable in the biological matrix or extracts if 80%-120% (QC1 (LLOQ)) or 85%-  
56 115% (QC3) of the reference concentration was recovered.  
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1 All stability experiments were performed in triplicate and results were expressed as mean ±  
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## 6 2.6 Clinical application 7

8 The developed assay was applied to saliva and plasma samples from a healthy adult  
9 volunteer participating in a pharmacokinetic study. The study was approved by the local  
10 Institutional Ethics Committee. Saliva and blood samples were collected at t=-30, -15 (saliva  
11 only), 20, 40, 60, 80, 100, 120, 150, 180, 210, 240, 300, 360 minutes following the intake of  
12 10 mg MPH-IR (Ritalin®). Following ingestion of 18 mg MPH-OROS (Concerta®), samples  
13 were collected at t=-30, -15 (saliva only), 20, 40, 60, 80, 100, 120, 150, 180, 210, 240, 270,  
14 300, 330, 360, 390, 420, 450, 480, 600 and 720 minutes. Study days were separated by at  
15 least 5 days to ensure complete wash-out. Blood samples were collected in EDTA tubes and  
16 put on ice immediately for 30 min. Saliva samples were obtained using the polyester  
17 Salivette swab system (Sarstedt AG, Nümbrecht, Germany). Samples were directly  
18 centrifuged at 2000 G for 10 minutes at 4°C and the plasma and saliva were stored at -80°C  
19 until analysis.  
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## 40 3. RESULTS AND DISCUSSION 41

### 42 3.1 Chromatography 43

44 Using electrospray in the positive mode MS parameters were tuned to produce maximum  
45 responses for MPH and the internal standard d<sub>9</sub>-MPH. The protonated molecular ions  
46 [M+H]<sup>+</sup> were *m/z* 234.1 and 243.1, respectively. The corresponding most abundant product  
47 ions were *m/z* 84.1 and 93.1.  
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55 The chromatographic results after injection of drug free plasma and saliva, a LLOQ sample  
56 and a patient receiving MPH are shown in Fig. 3. The chromatography shows excellent peak  
57 shape and symmetry, with a peak baseline resolution of less than 10 seconds. Under the  
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1 chromatographic conditions employed, the retention times were 24 s for MPH and 23 s for  
2 internal standard d<sub>9</sub>-MPH. The total runtime was 45 s. The reproducibility of the retention  
3 times was good for the several columns used during the development and validation of the  
4 method and the analysis of several thousand clinical samples (data not shown). The lifetime  
5 of the column was acceptable; more than 1000 injections could be made before  
6 chromatographic performance became unacceptable.  
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Marchei et al. [17] developed a reversed-phase chromatographic MS/MS method for MPH  
quantification with a runtime of 15 min. When using reversed phase chromatography, a short  
retention time of MPH is unfavorable since sensitivity may be reduced due to co-elution of  
matrix components. In the present study application of the HILIC column allowed a  
considerably shorter runtime.

### 3.2 Validation

#### 3.2.1 Selectivity

There were no discernable interfering components in commercially available and patient  
plasma and saliva. Fig. 3 shows chromatograms from blank plasma and saliva, plasma and  
saliva spiked with MPH at LLOQ and d<sub>9</sub>-MPH and a patient sample.

#### 3.2.2 Calibration

The calibration curves provided a linear response for the interval 0.5 – 100.0 µg/L. Un-  
weighted and weighted linear regression 1/x and 1/x<sup>2</sup> were compared by means of statistical  
and graphical methods. A weighting factor of 1/x provided the best fit. The value of each  
calibration standard was within 90-110% of the nominal value. The correlation coefficients  
(r<sup>2</sup>) of the 1/x-weighted calibration curves were in the range of 0.9997-1.0000 (n=6, mean  
0.9999) for plasma and in the range 0.9995-1.0000 (n=6, mean 0.9980) for saliva. The  
standard curves were y = 0.00791 (0.00130) x + 0.00235 (0.00306) for plasma and y =  
0.00807 (0.00130) x + 0.00051 (0.00043) for saliva (mean (95% CI); n=6). For plasma the

1 intercepts with the y-axis was not significantly different from zero, whereas a small but  
2 constant error was present for saliva.  
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### 6 *3.2.3 Accuracy and imprecision*

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8 The lower limit of quantification (LLOQ) for MPH was arbitrarily set at 0.5 µg/L (=QC1) in  
9 both plasma and saliva. The mean accuracy in both saliva and plasma was within the  
10 acceptance criteria of 85 – 115% for all QC levels (Table 1). For both plasma and saliva  
11 within-day and between-day imprecision were acceptable with values less than 7.0% in all  
12 QC samples. The mean accuracy of ten times diluted samples was acceptable as well.  
13 Accuracy was 110.5% and 100.2% for plasma samples with a concentration of 100 µg/L  
14 (QC3) and 1000 µg/L, respectively; the mean accuracy in saliva was 105.0% and 102.2% at  
15 similar concentrations.  
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### 29 *3.2.4 Process-efficiency and matrix effects*

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31 The process efficiency of the used method for the quantification of MPH in plasma was  
32 influenced by the occurrence of matrix effects. The matrix effects determined at plasma and  
33 saliva concentration of 10 µg/L were  $53.9 \pm 8.7\%$  and  $92.5 \pm 10.2\%$  (mean  $\pm$  SD, n=6),  
34 respectively, corresponding to 46.1% and 7.5% ion suppression. Matrix effects for d<sub>9</sub>-MPH  
35 were comparable:  $55.7 \pm 11.2\%$  for plasma and  $98.0 \pm 12.2\%$  for saliva. Notably, the matrix  
36 effect for d<sub>9</sub>-MPH in plasma was comparable, indicating the beneficial effect of using a  
37 deuterated internal standard. Recovery of MPH was  $116.6 \pm 6.3\%$  in plasma and  $103.4 \pm$   
38  $6.6\%$  in saliva; corresponding values for d<sub>9</sub>-MPH were  $113.8 \pm 7.7\%$  and  $99.8 \pm 7.7\%$  (mean  
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### 53 *3.2.5 Stability*

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55 At -80°C MPH concentrations in plasma decreased with  $6.4 \pm 3.1\%$  (QC1 (LLOQ)) and  $2.2 \pm$   
56  $1.9\%$  (QC3) after having been stored for 4 weeks (mean  $\pm$  SD, n=3). The corresponding  
57 values in saliva were  $2.4 \pm 1.0\%$  and  $0.3 \pm 0.7\%$ . Degradation at 4 weeks at -20°C was  $7.3 \pm$   
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1 4.2% for plasma and  $7.8 \pm 2.8\%$  for saliva in QC1; corresponding values for QC3 were  $2.4 \pm$   
2  $3.4\%$  and  $0.3 \pm 0.5\%$ . At  $-80^\circ\text{C}$  and  $-20^\circ\text{C}$ , stability was not studied for more than 4 weeks of  
3 storage. In QC1 plasma and saliva samples stored at  $5^\circ\text{C}$ ,  $43.8 \pm 6.1\%$  and  $42.7 \pm 3.1\%$  was  
4 degraded after two days, respectively. In QC3 plasma and saliva samples decay was  $39.1 \pm$   
5  $2.7\%$  and  $53.0 \pm 3.7\%$ . Apparently, degradation of MPH, caused by the catalytic activity of  
6 esterases, is still present at  $5^\circ\text{C}$ .  
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10 Figure 4 presents the degradation profile of MPH in plasma and saliva for QC1 and QC3 at  
11 room temperature. After 8 hours of storage the MPH concentration of MPH was decreased  
12 with  $5.4 \pm 4.2\%$  and  $7.0 \pm 4.9\%$  (mean  $\pm$  SD,  $n=3$ ) in the QC1 and QC3 plasma samples,  
13 respectively. After 1 hour saliva concentrations were reduced with  $13.2 \pm 3.5\%$  and  $6.9 \pm$   
14  $1.7\%$ , respectively. Half-life in plasma was  $81 \pm 7$  h and  $68 \pm 6$ h for QC1 and QC3,  
15 respectively; corresponding saliva values were  $24 \pm 4$ h and  $23 \pm 3$ h (mean  $\pm$  SD,  $n=3$ ).  
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26 The degradation of QC3 plasma and saliva samples after 3 freeze/thaw cycles was  
27 acceptable; corresponding values were  $6.4 \pm 4.2\%$  and  $12.4 \pm 9.3\%$  (mean  $\pm$  SD,  $n=3$ ). For  
28 QC1 samples significant degradation was observed in plasma after the second cycle ( $38.4 \pm$   
29  $9.2\%$ ) and in saliva after the third cycle ( $18.4 \pm 6.2\%$ ). This indicates at lower concentrations  
30 the number of freeze/thaw cycles should be limited to 1.  
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37 The processed plasma and saliva samples were stable in the autosampler ( $10^\circ\text{C}$ ) for 24  
38 hours, suggesting that all esterase activity is eliminated after protein precipitation.  
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42 Little information is available in literature on the stability of MPH in plasma and saliva.  
43 Considering the present results, plasma and saliva samples should be immediately frozen at  
44  $-20^\circ\text{C}$  after collection from the patient. Protein precipitation should be performed directly  
45 following thawing of the sample.  
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### 53 *3.3 Clinical application*

54 The developed method was successfully applied for the assessment of MPH concentration  
55 profiles in plasma and saliva in an adult healthy volunteer taking 10 mg MPH as IR  
56 preparation and 18 mg as OROS (Figure 5). The time profile of MPH concentration in saliva  
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1 followed more or less the plasma time profile. During the first hour after ingestion of the IR  
2 preparation, saliva concentrations were approximately two-fold higher than plasma  
3 concentrations. This may be caused by some degree of dissolution of the IR formulation in  
4 the mouth, as this was given in its commercially available tablet form. Two-fold higher saliva  
5 concentrations were also observed following ingestion of the OROS formulation, which is a  
6 capsule, indicating that another mechanism influencing the distribution between plasma and  
7 saliva may be involved as well. MPH is an amphetamine-like compound that has low plasma  
8 protein binding (approximately 15%), and low molecular weight (233 Da) and shows the  
9 characteristics of a weak base ( $pK_a = 8.9$ ). Based on these characteristics, ion trapping may  
10 occur, as has also been described for methylenedioxymethamphetamine (MDMA) [23]. The  
11 free MPH fraction passively distributes in its ionized form from blood to saliva (which is more  
12 acidic than blood) and then cannot diffuse back into plasma, leading to higher MPH  
13 concentrations in saliva compared to those in plasma.  
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#### 31 **4. CONCLUSION**

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35 A LC-MS/MS method using hydrophilic interaction liquid chromatography has been  
36 successfully developed for determination of MPH concentrations in plasma and saliva. The  
37 method has proven to be rapid, sensitive, accurate and precise. Due to matrix effects of  
38 plasma, the use of deuterated MPH as an internal standard was essential. Stability  
39 experiments demonstrated that samples should be stored at temperatures of  $-20^{\circ}\text{C}$  or below  
40 directly after sampling, and that samples should be processed immediately after thawing.  
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42 The potential use of the assay for therapeutic monitoring of MPH concentrations in saliva  
43 looks promising, but needs further investigation.  
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## HIGHLIGHTS

- A LC-MS/MS method was validated for the determination of methylphenidate (MPH) in plasma and saliva.
- The method allows fast, accurate and precise quantification of MPH in both plasma and saliva.
- The use of deuterated MPH as an internal standard is essential, due to matrix effects of plasma.
- MPH is degraded in plasma and saliva; clinical samples should be stored at -20°C directly after sampling.

## CAPTIONS

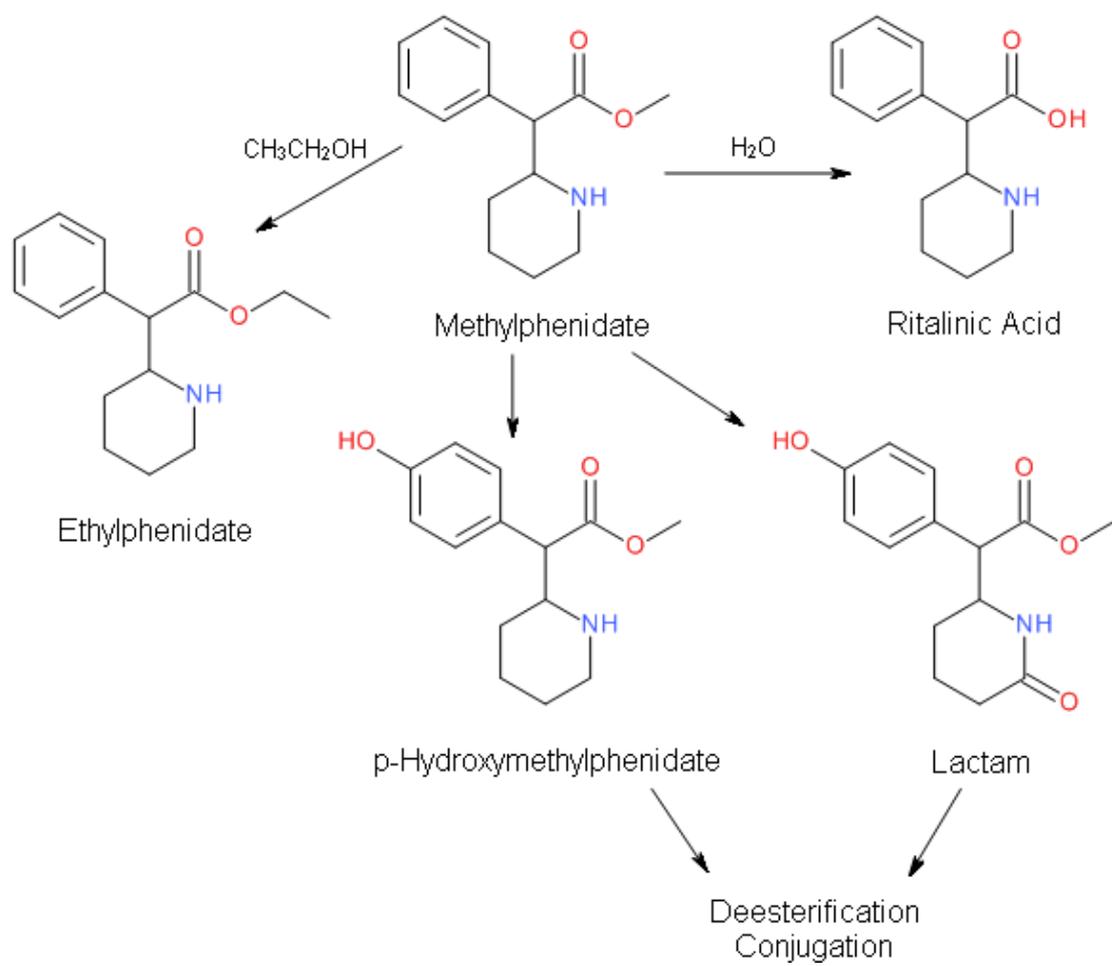
Figure 1. Metabolism of methylphenidate

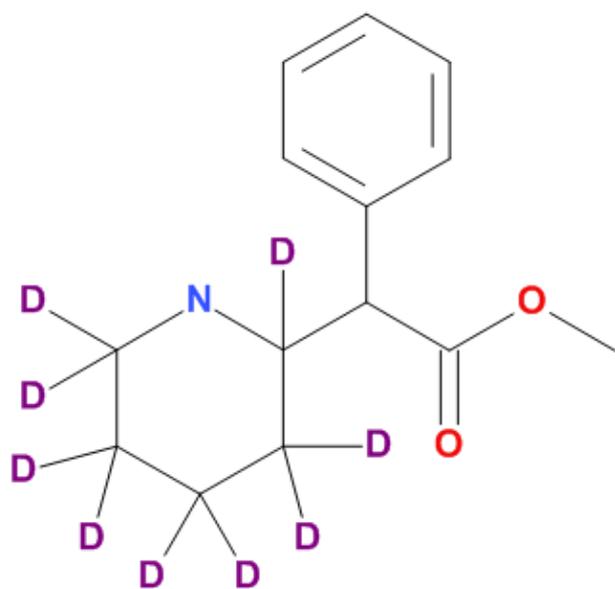
Figure 2. Molecular structure of d<sub>9</sub>-methylphenidate

Figure 3. Chromatograms of blanco plasma (A), spiked plasma with a methylphenidate concentration of 5 µg/L (LLOQ) (B) and patient plasma (C). Chromatograms of blanco saliva (D), spiked saliva with a methylphenidate concentration of 0.5 µg/L (LLOQ) (E) and patient saliva (F).

Figure 4. Degradation of methylphenidate in plasma (squares) and saliva (circles) at room temperature. Closed and open symbols represent the concentration at 0.5 µg/L (QC1 (LLOQ)) and 100 µg/L (QC3), respectively (mean ± SD, n=3). The fitted lines represent the fitted first-order decay for a concentration of 0.5 µg/L (dashed line) and 100 µg/L (solid line).

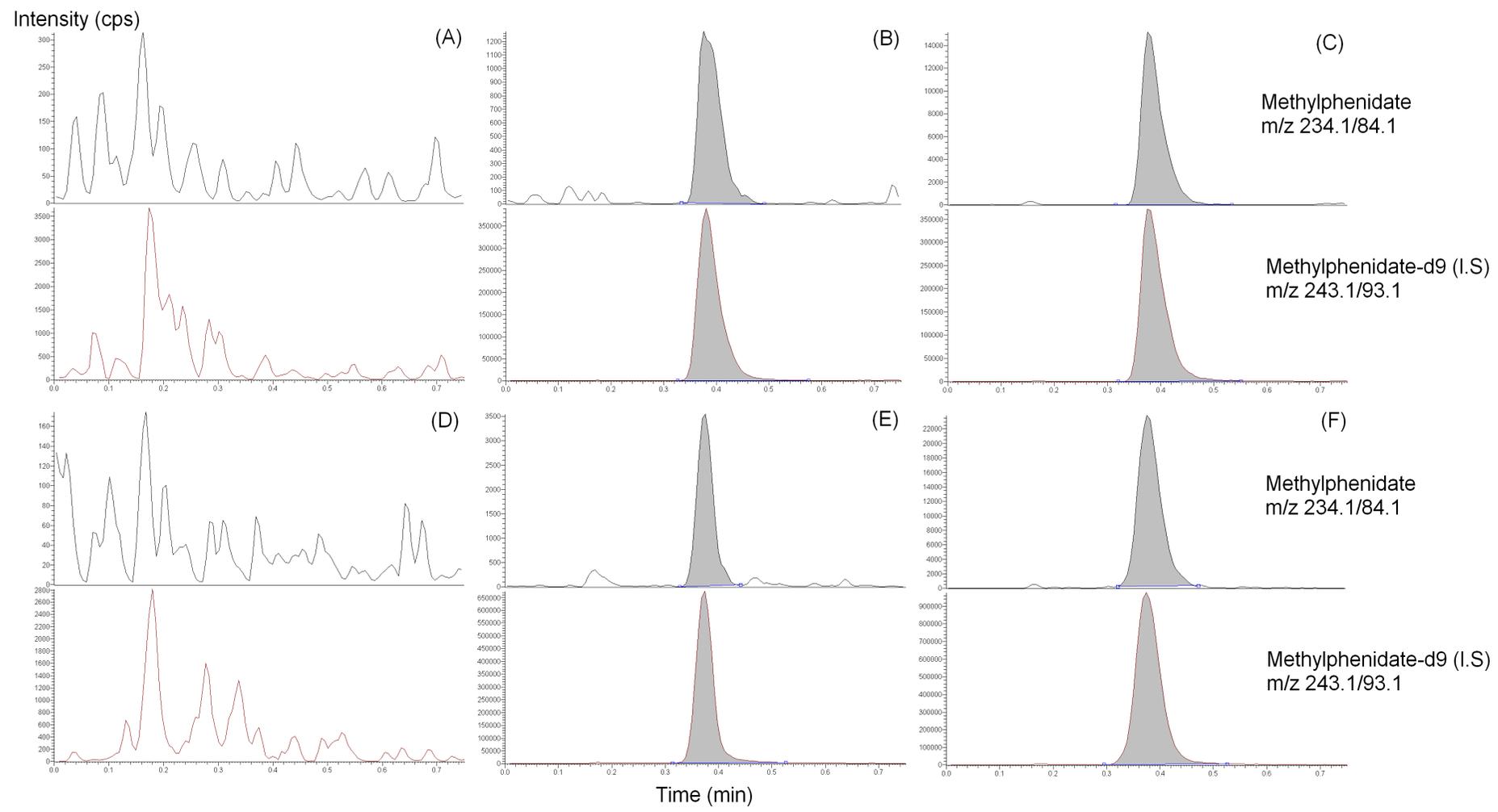
Figure 5. Time profiles of methylphenidate concentration in plasma (closed squares) and saliva (open squares) in a healthy adult volunteer after intake of 10 mg methylphenidate (MPH) in an immediate release formulation (Ritalin®; top) and 18 mg MPH in a sustained release preparation (Concerta®; bottom) on different occasions

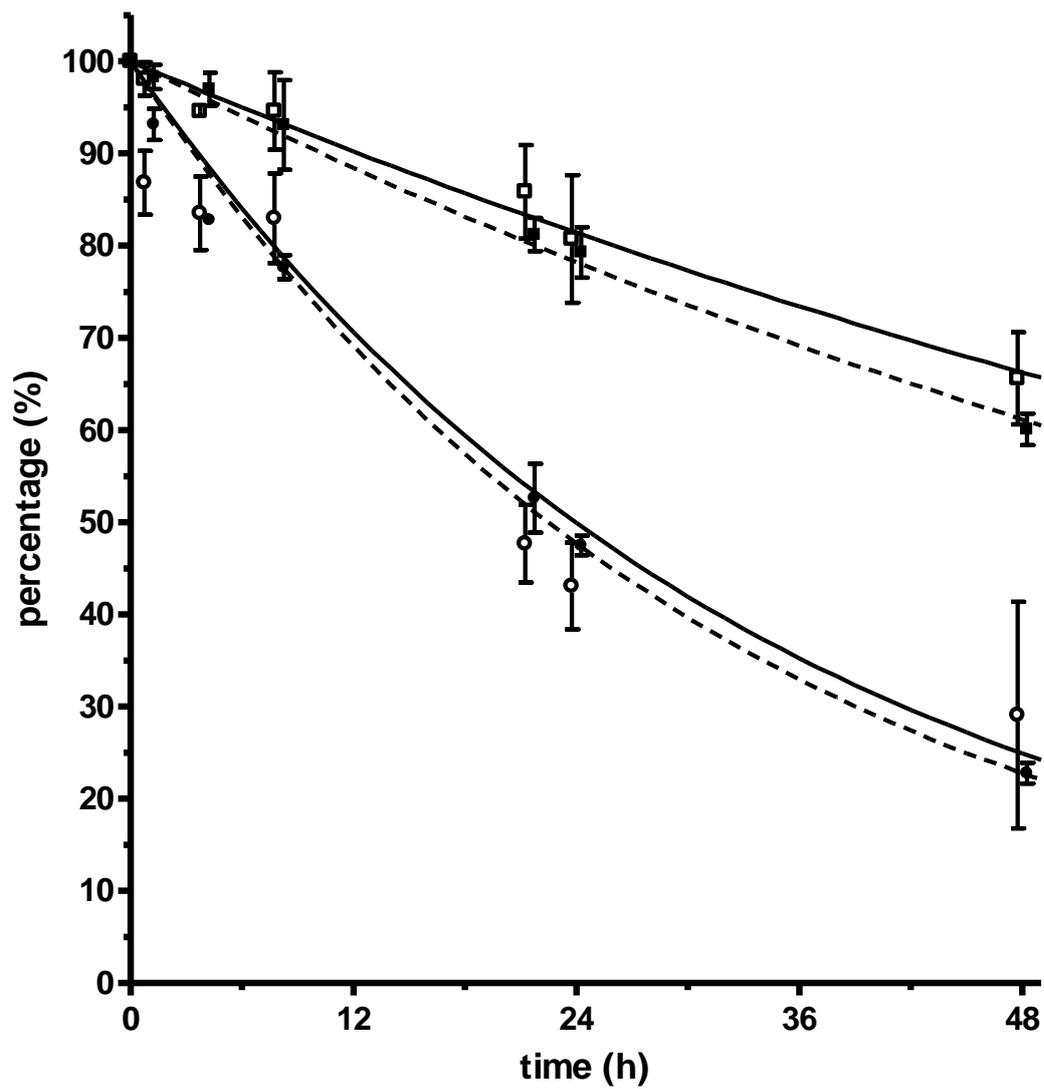




**Figure 3**

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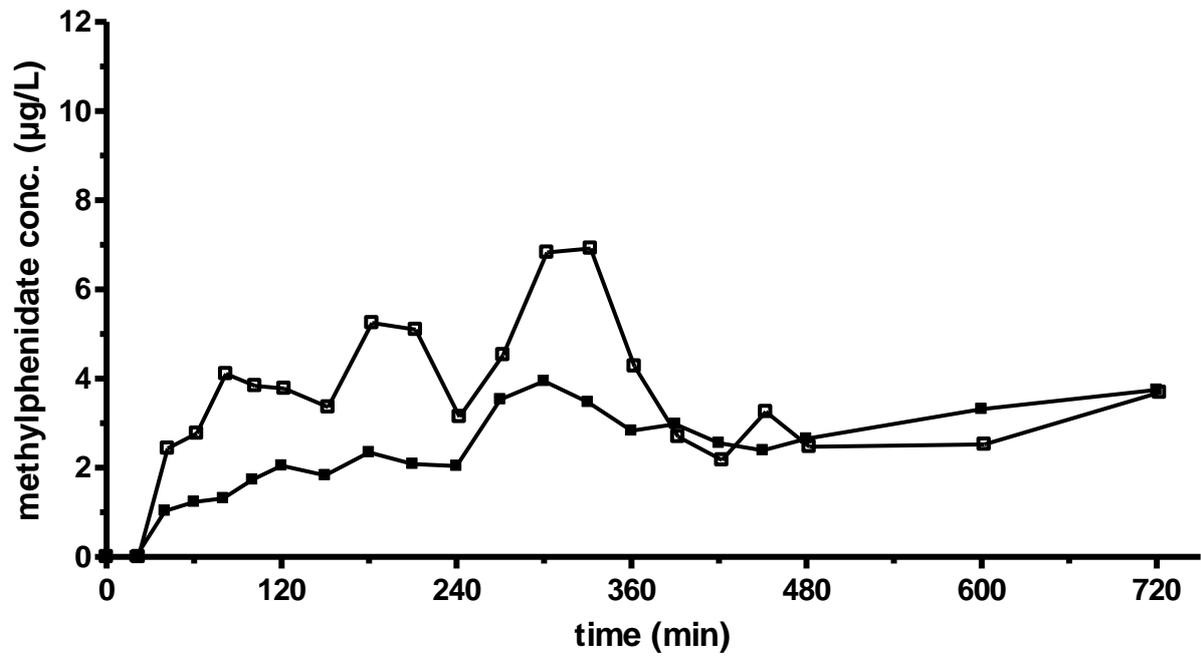
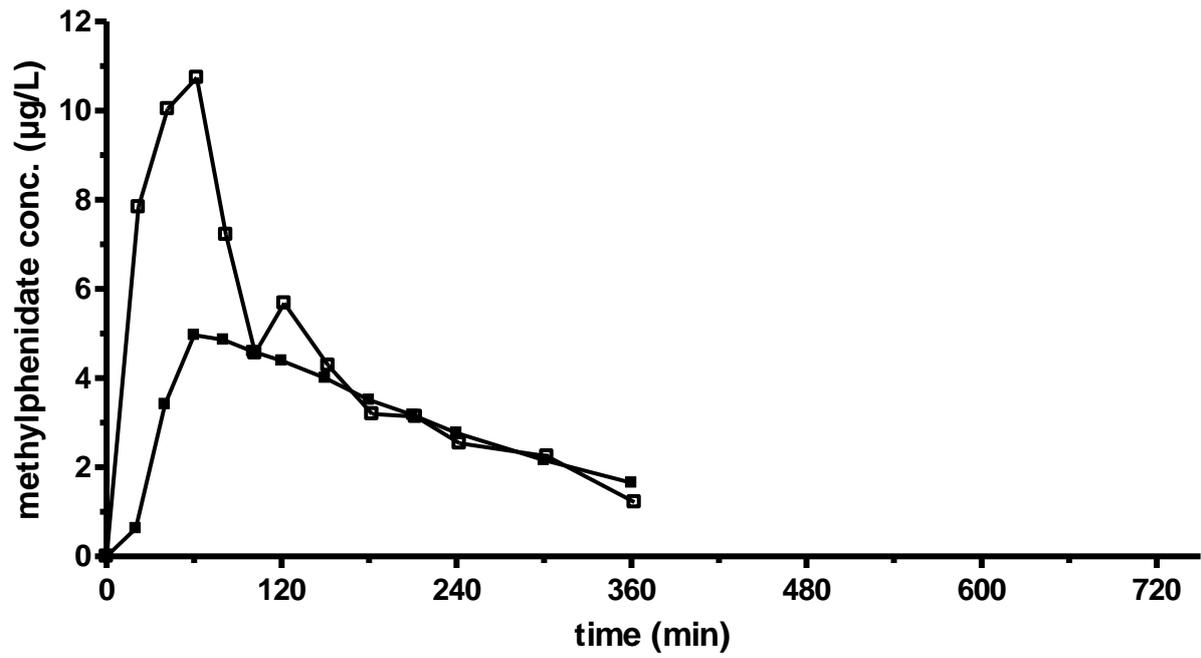


Table 1. Accuracy, within-day and between day imprecision for the determination of MPH in plasma and saliva (n=6)

Plasma				
QC sample	Concentration (µg/L)	Accuracy (%)	Within-day imprecision (%)	Between-day imprecision (%)
QC1 (LLOQ)	0.5	107.8	5.0	6.9
QC2	10	101.1	7.0	1.6
QC3	100	101.3	4.5	1.7
Saliva				
QC sample	Concentration (µg/L)	Accuracy (%)	Within-day imprecision (%)	Between-day imprecision (%)
QC1 (LLOQ)	0.5	106.2	5.9	5.4
QC2	10	99.3	2.2	4.2
QC3	100	98.3	3.4	2.9