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Non-invasive monitoring of pharmacokinetics and pharmacodynamics for pharmacological drug profiling in children and adolescents
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CHAPTER 4

Determination of methylphenidate in plasma and saliva by liquid chromatography/tandem mass spectrometry

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ABSTRACT

Methylphenidate (MPH) is a phenethylamine derivative used in the treatment of attention-deficit hyperactivity disorder (ADHD). In adults, clinical monitoring of MPH therapy is usually performed by measuring plasma MPH concentrations. In children blood sampling is however undesirable. Saliva may be an alternative matrix for monitoring MPH concentrations with the advantage that it can be obtained non-invasively. Therefore, we developed an analytical method for the quantification of MPH in both plasma and saliva.

We present the validation of a liquid chromatography-tandem mass spectrometric method using a hydrophilic interaction liquid chromatography column (HILIC). In 100 μl sample, proteins were precipitated with 750 μl acetonitrile/methanol 84/16 (v/v) containing d_9-methylphenidate as the internal standard. Standard curves were prepared over the MPH concentration range of 0.5-100.0 μg/L. The total analysis time was 45 seconds. Accuracy and within- and between-run imprecision were in the range of 98-108% and less than 7.0%, respectively. Matrix effects were greater for plasma than saliva with 46% and 8% ionization suppression. The matrix effects were adequately compensated by the use of deuterated MPH as internal standard. MPH significantly degraded in plasma and saliva at room temperature and 5°C. Samples were stable at -20°C for at least 4 weeks. The method was successfully applied for the determination of MPH concentrations in plasma and saliva samples from an adult healthy volunteer. Using protein precipitation and hydrophilic interaction liquid chromatography coupled to tandem mass spectrometry, this method allows fast, accurate and precise quantification of MPH in both plasma and saliva.

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[1,2]. MPH is a piperidine-derived molecule that contains two chiral centers and exists as four stereoisomers (Figure 1). The pharmacological activity resides entirely with the *dl-threo*-methylphenidate racemic (50:50) mixture[3].

The major metabolic pathway of MPH is the hydrolysis of the methyl ester linkage by esterases to form ritalinic acid[4-5]. Minor metabolic pathways for both these compounds include parahydroxylation of the aromatic ring, oxidation to 6-oxo-dervatives and glucuronide formation[6-7]. Ritalinic acid and the other metabolites are pharmacologically inactive[8-10].

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[11-12]. 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 as demonstrated earlier by Marchei and coworkers[13].

Several methods have been developed for quantification of MPH in plasma, urine and hair, using high-performance liquid chromatography (HPLC) with ultraviolet detection[6,14], capillary electrophoresis-mass spectrometry[15], gas chromatography-mass spectrometry[8,9,16] and liquid chromatography–tandem mass spectrometry[7,13,17-18]. The determination of MPH concentrations by standard reversed-phase (RP) chromatography coupled to MS/MS detection is particularly challenging since retention times may be short due to the high hydrophilicity of the compound. This may produce a significant loss in sensitivity due to the co-elution with matrix interference and the high percentage of water at the chromatographic elution time. Recent research however has shown that for hydrophilic compounds the sensitivity, precision and accuracy of a quantitative analytical chromatographic method may be improved by using hydrophilic interaction liquid chromatography (HILIC)[19]. In addition, the use of HILIC has advantages in sample preparation when measuring polar compounds. Because of the high organic modifier content, usually acetonitrile, used during chromatography, proteins can be precipitated using organic solvents without the loss of chromatographic integrity, as is often the case when used with polar compounds in combination with RP chromatography. A high organic modifier concentration is also ideal for compound ionization by electrospray ionization mass spectrometry (ESI-MS), resulting in higher sensitivity.

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

Materials and methods

Chemicals

Methylphenidate was purchased from Bufa (Uitgeest, Netherlands). As an internal standard (I.S.) a 1 mg/ml solution of deuterated methylphenidate HCl (d₉-MPH) in methanol was obtained from LGC-Standards (Teddington, United

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Kingdom) (Figure 1). Water was purified and deionized using an ELGA purelab Optron Q (Veolia Water; Saint Maurice, France). Drug free, non sterile, K$_2$ EDTA human plasma was obtained from Equitech-Bio (Kerrville-TX, USA). OraFlx synthetic saliva was obtained from Dyna-Tek (Lenexa-KS, USA).

Instrumentation

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

LC-MS/MS conditions

Isocratic elution was applied using A: 2% formic acid in water (v/v) and B: acetonitrile 100%. A was set at 10% and B at 90%. Analytical separation was accomplished on a SeQuant ZIC-HILIC column (Merck, Darmstadt, Germany) with a length of 50 mm, an internal diameter of 2.1 mm and 5 µm particle size. The flow rate was 1.00 ml/min giving a total chromatographic run time of only 45 seconds. To minimize carry-over effects the LC injection system was washed with 20% formic acid in water (v/v) after every injection. The autosampler temperature was maintained at 10°C, the column oven at 30°C. The analytes were detected in positive ion mode using multiple reaction monitoring (MRM). The ion spray voltage was 5000 V and the ion transfer tube temperature was 250°C. Sheath and auxiliary gas pressure were 50 and 20 psi, respectively. Collision gas (argon) pressure was 2.0 mTorr. MPH and d$_9$-MPH were measured as [M+H]$^+$ using the mass transitions 234.1 → 84.1 and 243.1 → 93.1 respectively. Tube lens voltage and collision energy were 90 and 21 V, respectively. Dwell time was 300 ms for MPH and 50 ms for d$_9$-MPH.

Analytical procedures

PREPARATION OF STOCK SOLUTIONS, CALIBRATION STANDARDS AND QUALITY CONTROL SOLUTIONS

Two stock MPH solutions (5.0 mg/L and 50.0 mg/L) were prepared by dissolving MPH in water/methanol 1/1 (v/v). The stock internal standard (10 µg/L) was prepared by diluting the 1 mg/ml d$_9$-MPH standard solution with acetonitrile/methanol 84/16 (v/v). All stock solutions were stored at 5°C until use.

Four MPH calibration standard solutions (5.0, 10.0, 25.0 and 50.0 µg/L) were prepared by diluting 10, 20, 50, and 100 µl of the 5.0 mg/L stock solution in 10 ml of water/methanol 1/1 (v/v). Three MPH calibration standard solutions (100, 400 and 1000 µg/L) were prepared by diluting 20, 80 and 200 µl of the 50.0 mg/L stock solution in 10 ml of water/methanol 1/1 (v/v). 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 µg/L. The calibration standard and QC solutions were stored at 5°C until use.

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 µl was pipetted into a 1.8 ml vial. Subsequently, 100 µl 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 µg/L and final concentrations of the quality controls were 0.5 (QC1 (LLOQ)), 10.0 (QC2) and 100.0 µg/L (QC3). Patient plasma and saliva samples were thawed and shortly vortexed and 100 µl of each sample was pipetted into a 1.8 ml vial. Subsequently, 10 µl of water/methanol 1/1 (v/v) was added and shortly vortexed. In all samples proteins were precipitated by adding 750 µl of the internal standard solution. After vortexing for 1 minute,

samples were stored at -20°C for 30 minutes to enhance protein precipitation, vortexed again and centrifuged for 5 minutes at 480x*g*. Two microliter of the supernatant was injected.

QUANTIFICATION

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

Method validation

SELECTIVITY

One lot of blank, commercially acquired saliva and plasma, together with saliva and plasma samples from five different patients, not receiving MPH, were tested for interferences. Proteins were precipitated using acetonitrile/methanol 84/16 (v/v) without I.S. The data of the chromatograms were processed and the integrated response should not exceed 10% of the average integrated response of the LLOQ of MPH and 1% of the integrated response of d_9-MPH.

CALIBRATION

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

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 Adminstration 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%[19]. 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[20].

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%.

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[21-22]. Assay recovery and matrix effects were quantified for both plasma and saliva using the strategies proposed by Matuszewski *et al*[23]. 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_9-MPH concentrations were 10 µg/L. Recovery (RE) was defined as the relative signal of samples spiked post-precipitation versus pre-precipitation. Matrix effects (ME) were similarly defined as the relative signal of post-precipitation spiked plasma and saliva samples versus spiked aqueous samples. A value of 100% for ME

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indicated that signals in plasma/saliva samples and aqueous samples phase were similar. A ME value greater than 100% indicated ionization enhancement, whereas a value less than 100% indicates ionization suppression.

Process efficiency (PE) was defined as the product of RE and ME, i.e. the overall signal of spiked plasma and saliva versus an aqueous standard solution. Average values and coefficients of variation of RE, ME and PE were calculated over the six plasma and saliva batches.

STABILITY

The stability of MPH in saliva and plasma QC1 (LLOQ) and QC3 samples was determined for several storage conditions. The freeze-thaw stability in plasma and saliva was determined by comparing freshly prepared samples with samples that underwent three freeze-thaw cycles (24 hours at -80°C). The MPH concentration of plasma and saliva samples stored at -20°C and -80°C was determined weekly and compared with freshly prepared samples. The stability of MPH in plasma and saliva at 5°C was assessed after 2, 5 and 7 days of storage. The time course of MPH degradation in plasma and saliva was studied at room temperature by determination of the MPH concentration at the start of the experiment and 1, 4, 8, 21.5, 24 and 48 hours after the start. The esterase mediated decay of MPH in plasma and saliva was described by a first-order process. Data were log-transformed and rate constants were obtained by linear regression. Half life was calculated by dividing 0.693 by the rate constant.

The MPH concentration of processed samples stored in the autosampler (10°C) was determined after 24 hours and compared with the initial concentration. The analyte was considered stable in the biological matrix or extracts if 80%-120% (QC1 (LLOQ)) or 85%-115% (QC3) of the reference concentration was recovered.

All stability experiments were performed in triplicate and results were expressed as mean ± SD.

Clinical application

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

Results and discussion

Chromatography

Using electrospray in the positive mode MS parameters were tuned to produce maximum responses for MPH and the internal standard d$_9$-MPH. The protonated molecular ions [M+H]$^+$ were m/z 234.1 and 243.1, respectively. The MS2 spectra of both methylphenidate and d$_9$-methylphenidate are given in Figure 2; the corresponding most abundant product ions were m/z 84.1 and 93.1.

The chromatographic results after injection of drug free plasma and saliva, a LLOQ sample and a patient receiving MPH are shown in Figure 3. The chromatography shows excellent peak shape and symmetry, with a peak baseline

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resolution of less than 10 seconds. Under the chromatographic conditions employed, the retention times were 24 s for both MPH and internal standard d_9-MPH. The total runtime was 45 s. The reproducibility of the retention times was good for the several columns used during the development and validation of the method and the analysis of several thousand clinical samples (data not shown). The lifetime of the column was acceptable; more than 1000 injections could be made before chromatographic performance became unacceptable.

Total runtimes of reversed-phase chromatographic MS/MS methods for MPH quantification have been reported to range from 5 to 15 min[18, 24-25]. When using reversed phase chromatography, a short retention time of MPH may be unfavorable since sensitivity may be reduced due to co-elution of matrix components. In the present study application of the HILIC column allowed a total runtime of 45 s, which was at least 6-fold shorter than published reversed-phase methods[18, 24-25].

Methods have been developed for the simultaneous determination of MPH and its inactive metabolite ritalinic acid[18, 24-25]. Assesment of the latter may be particularly useful in forensic studies or compliance studies since ritalinic acid concentrations are generally 10- to 50- fold higher than methylphenidate concentrations and RA may be detected longer following ingestion due to its longer elimination half-life[26]. In case of therapeutic drug monitoring detection of ritalinic acid has no value, since the individual dose is adjusted on basis of determined concentrations of methylphenidate only.

Validation

SELECTIVITY

There were no discernable interfering components in commercially available and patient plasma and saliva. Figure 3 shows chromatograms from blank plasma and saliva, plasma and saliva spiked with MPH at LLOQ and d_9-MPH and a patient sample.

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/x2 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^2) 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 intercepts with the y-axis was not significantly different from zero, whereas a small but constant error was present for saliva.

ACCURACY AND IMPRECISION

The lower limit of quantification (LLOQ) for MPH was arbitrarily set at 0.5 µg/L (=QC1) in both plasma and saliva. Only noise was detected when blank plasma and saliva samples were injected following the injection of the highest calibration standard; carry-over was less than 0.1%.

The mean accuracy in both saliva and plasma was within the acceptance criteria of 85 – 115% for all QC levels (Table 1). For both plasma and saliva within-day and between-day imprecision were acceptable with values less than 7.0% in all QC samples. The mean accuracy of ten times diluted samples was acceptable as well. Accuracy was 110.5% and 100.2% for plasma samples with a concentration of 100 µg/L (QC3) and 1000 µg/L, respectively; the mean accuracy in saliva was 105.0% and 102.2% at similar concentrations.

PROCESS-EFFICIENCY AND MATRIX EFFECTS

The process efficiency of the used method for the quantification of MPH in plasma was influenced by the occurrence of matrix effects. The matrix effects determined at plasma and saliva concentration of 10 µg/L were 53.9 ± 8.7% and

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92.5 ± 10.2% (mean ± SD, n=6), respectively, corresponding to 46.1% and 7.5% ion suppression. Matrix effects for d_9-MPH were comparable: 55.7 ± 11.2% for plasma and 98.0 ± 12.2% for saliva. Notably, the matrix effect for d_9-MPH in plasma was comparable, indicating the beneficial effect of using a deuterated internal standard. Recovery of MPH was 116.6 ± 6.3% in plasma and 103.4 ± 6.6% in saliva; corresponding values for d_9-MPH were 113.8 ± 7.7% and 99.8 ± 7.7% (mean ± SD, n=6). Wang and colleagues reported that a slight difference in retention time between analyte and an internal standard labeled with a stable isotope may cause a different degree of ion suppression between the analogues and, consequently, influence the accuracy of the method[27]. In our method retention times of MPH and d_9-MPH were similar and >90% of the peak areas were overlapping. As a result, patient- and time-dependent variability in ion suppression will not affect the accuracy of our method.

STABILITY

At -80°C MPH concentrations in plasma decreased with 6.4 ± 3.1% (QC1 (LLOQ)) and 2.2 ± 1.9% (QC3) after having been stored for 4 weeks (mean ± SD, n=3). The corresponding values in saliva were 2.4 ± 1.0% and 0.3 ± 0.7%. Degradation at 4 weeks at -20°C was 7.3 ± 4.2% for plasma and 7.8 ± 2.8% for saliva in QC1; corresponding values for QC3 were 2.4 ± 3.4% and 0.3 ± 0.5%. At -80°C and -20°C, stability was not studied for more than 4 weeks of storage. In QC1 plasma and saliva samples stored at 5°C, 43.8 ± 6.1% and 42.7 ± 3.1% was degraded after two days, respectively. In QC3 plasma and saliva samples decay was 39.1 ± 2.7% and 53.0 ± 3.7%. Apparently, degradation of MPH, caused by the catalytic activity of esterases, is still present at 5°C.

Figure 4 presents the degradation profile of MPH in plasma and saliva for QC1 and QC3 at room temperature. After 8 hours of storage the MPH concentration of MPH was decreased with 5.4 ± 4.2% and 7.0 ± 4.9% (mean ± SD, n=3) in the QC1 and QC3 plasma samples, respectively. After 1 hour saliva concentrations were reduced with 13.2 ± 3.5% and 6.9 ± 1.7%, respectively. Half-life in plasma was 81 ± 7h and 68 ± 6h for QC1 and QC3, respectively; corresponding saliva values were 24 ± 4h and 23 ± 3h (mean ± SD, n=3).

The degradation of QC3 plasma and saliva samples after 3 freeze/thaw cycles was acceptable; corresponding values were 6.4 ± 4.2% and 12.4 ± 9.3% (mean ± SD, n=3). For QC1 samples significant degradation was observed in plasma after the second cycle (38.4 ± 9.2%) and in saliva after the third cycle (18.4 ± 6.2%). This indicates at lower concentrations the number of freeze/thaw cycles should be limited to 1.

The processed plasma and saliva samples were stable in the autosampler (10°C) for 24 hours, suggesting that all esterase activity is eliminated after protein precipitation.

Little information is available in literature on the stability of MPH in plasma and saliva. Considering the present results, plasma and saliva samples should be immediately frozen at -20°C after collection from the patient. Protein precipitation should be performed directly following thawing of the sample.

Clinical application

The developed method was successfully applied for the assessment of MPH concentration profiles in plasma and saliva in an adult healthy volunteer taking 10 mg MPH as IR preparation and 18 mg as OROS (Figure 5). The time profile of MPH concentration in saliva followed more or less the plasma time profile. During the first hour after ingestion of the IR preparation, saliva concentrations were approximately two-fold higher than plasma concentrations. This may be caused by some degree of dissolution of the IR formulation in the mouth, as this was given in its commercially available tablet form. Two-fold higher saliva concentrations were also observed following ingestion of the OROS formulation, which is a capsule, indicating that another mechanism influencing the distribution between plasma and saliva may be involved as well. MPH is an ampetamine-like compound that has low plasma protein binding (approximately 15%), and low molecular weight (233 Da) and shows the characteristics of a weak base (pK_a = 8.9). Based on these characteristics, ion trapping may occur, as has also been described for methylenedioxymethamphetamine (MDMA)[28]. The free MPH fraction passively distributes in its ionized

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form from blood to saliva (which is more acidic than blood) and then cannot diffuse back into plasma, leading to higher MPH concentrations in saliva compared to those in plasma.

Alternative biological matrices (hair, oral fluid, sweat) have been studied earlier for monitoring therapeutic use or misuse of methylphenidate[18, 29]. Marchei and coworkers demonstrated that the overall patterns of concentration-time profiles of plasma and saliva MPH agreed reasonably well following the administration of fast- and extended-release formulations[13]. They reported higher saliva concentrations than plasma concentrations, which is in accordance with the result of the present study. In the referred study however the saliva/plasma ratio proved to be time- and formulation dependent, which may have hampered clinical application of saliva monitoring so far.

Conclusion

An LC-MS/MS method using hydrophilic interaction liquid chromatography has been successfully developed for determination of MPH concentrations in plasma and saliva. The method has proven to be rapid, sensitive, accurate and precise. Due to matrix effects of plasma, the use of deuterated MPH as an internal standard was essential. Stability experiments demonstrated that samples should be stored at temperatures of -20°C or below directly after sampling, and that samples should be processed immediately after thawing. The assay allows further investigation of therapeutic monitoring of MPH concentrations in saliva.

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

	qc sample	Concentration (µg/L)	Accurcay (%)	Within-day imprecision (%)	Between-day imprecision (%)
Plasma	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	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

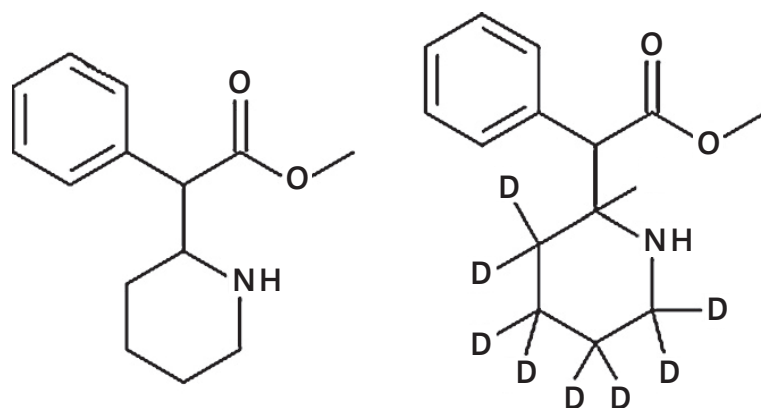**FIGURE 1**   Molecular structure of methylphenidate (left) and d9-methylphenidate (right).

FIGURE 2   Product ion mass (MS2) spectra of [M+H]+ for methylphenidate (top) and [M+H]+ for d9-methylphenidate (bottom).

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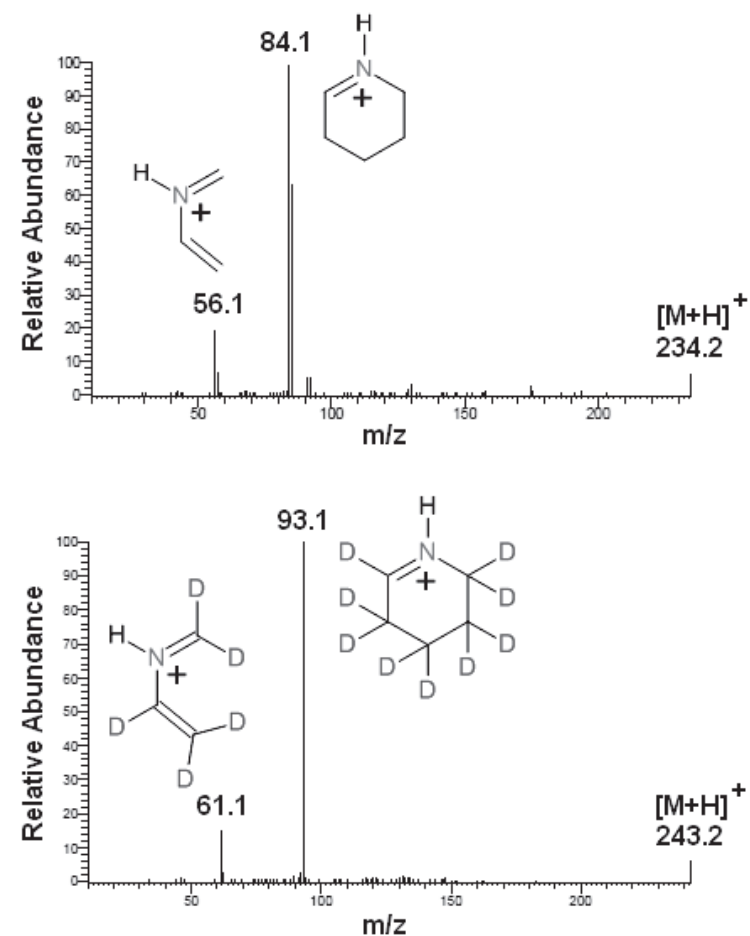CHAPTER 4 — 4 MPH DETERMINATION IN PLASMA AND SALIVA BY LC-MS/MS
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FIGURE 3 Chromatograms of blanco plasma (A), spiked plasma with a methylphenidate concentration of 5 µg/L (LLOQ) (B) and patient plasma (C). Chromatograms of blanco saliva (D), spiked saliva with a methylphenidate concentration of 0.5 µg/L (LLOQ) (E) and patient saliva (F).
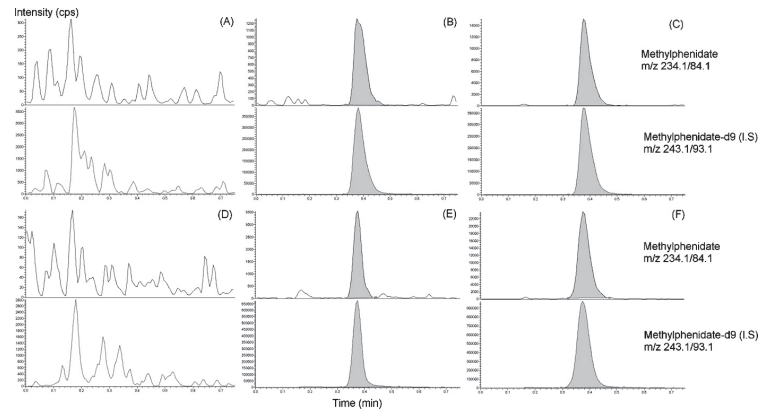

FIGURE 4 Degradation of methylphenidate in plasma (squares) and saliva (circles) at room temperature. Closed and open symbols represent the concentration at 0.5 µg/L (QC1 (LLOQ)) and 100 µg/L (QC3), respectively (mean ± SD, n = 3). The fitted lines represent the fitted first-order decay for a concentration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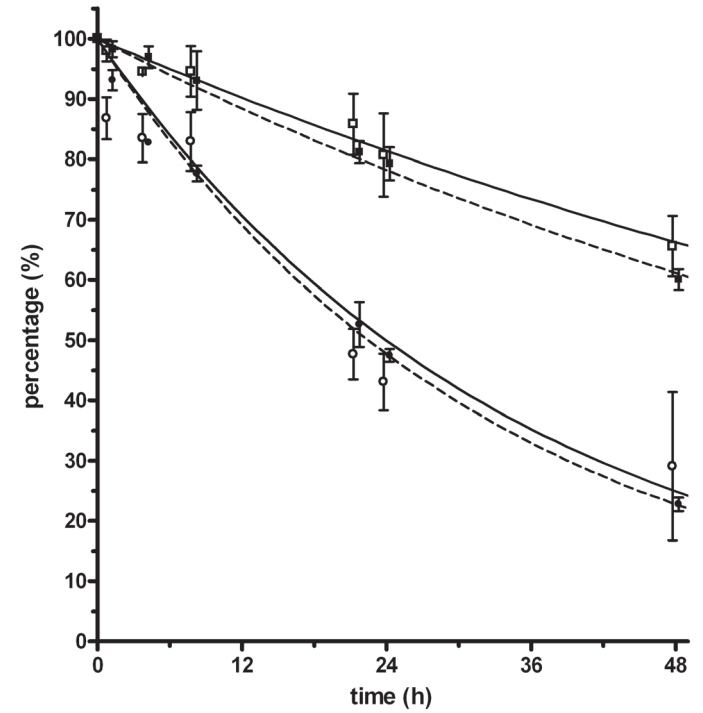

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