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Gas chromatography–negative ion chemical ionisation mass spectrometry using *o*-(pentafluorobenzyloxycarbonyl)-2,3,4,5-tetrafluorobenzoyl derivatives for the quantitative determination of methylphenidate in human plasma

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ABSTRACT

A novel electrophoric derivatisation procedure using *o*-(pentafluorobenzyloxycarbonyl)-2,3,4,5-tetrafluorobenzoyl chloride for the quantitative determination of methylphenidate in human plasma is described. The drug can be quantitatively measured down to 0.006 pg/mL plasma due to the extraordinary sensitivity of the derivatives under negative ion chemical ionisation mass spectrometry. Plasma samples were made alkaline with carbonate buffer and treated with extraction solvent (n-hexane) and reagent solution for 15 min, which, after concentration was measured by GC–NICI–MS. The method is rapid as extraction and derivatisation occur in one single step. A stable isotope labelled internal standard was used. Validation data are given to demonstrate the usefulness of the assay, including selectivity, linearity, accuracy and precision, autosampler stability, aliquot analysis, robustness, and prospective analytical batch size accuracy. The method has been successfully applied to pharmacokinetic profiling of the drug after oral administration.

1. Introduction

Attention-deficit hyperactive disorder (ADHD) shows a high prevalence of 5–10% of the general population [1,2]. It is a neurobehavioural problem mostly encountered with school-age children. Methylphenidate [*dl-threo*-methyl 2-phenyl-2-(piperidyl)actetate] (MPH), a cyclic amphetamine analogue, is widely used for the treatment of children having ADHD, with or without hyperactivity [3]. MPH is a psychostimulant drug that displays a high intrinsic clearance due to the rapid stereoselective hydrolysis of the methyl ester function [4,5]. As a consequence, the plasma concentrations after oral therapeutic doses encountered are low, typically witn c_{max} of 10–20 ng/mL. For optimal medication and elimination of toxicological side effects it is necessary to adjust drug dosage individually as there is a large interindividual variability in the response to MPH concentrations. This is of particular importance as children are the main target group of this medication [6]. Quantification in the lower picogram/mL range is hence desirable for reliable pharmacokinetic studies with MPH. Besides for the treatment of ADHD, MPH improves attention, concentration, fine motor coordination and balance and due to these sport-related benefits the drug is considered as a doping agent [7,8]. For instance, CNS stimulant medication used in the management of ADHD is not permitted for use in competition by the International Olympic Committee (IOC).

Several analytical methods have been reported for the determination of MPH in plasma and urine. High-performance liquid chromatography (HPLC) has been used with fluorescence detection [9] as well as in combination with mass spectrometry (MS) [10–14] and tandem MS [15]. Heptafluorobutyryl-L-prolyl and trifluoroacety-L-prolyl derivatives were used for enantioselective measurement of the drug by GC–MS using either EI [16], positive ion chemical ionization (PICI) [17] or negative ion chemical ionization (NICI) [17,18]. Besides that, GC–MS of pentafluoropropyl derivatives have been used with electron ionization (EI) [19,20] and positive ion chemical ionisation [21], and more recently capillary electrophoresis ion-trap mass spectrometry [22]. Besides the advantage of enantioselective detection, none of the methods achieved a limit of quantification (LOQ) below 0.4 ng/mL. A sensitive method has been described to detect 70 pg/mL plasma by using GC–negative ion chemical ionisation (NICI)–MS of the heptafluorobutyrate [23].

We have recently introduced a new derivatisation reagent, *o*-(pentafluorobenzyloxycarbonyl)-2,3,4,5-tetrafluorobenzoyl chloride (PBTFBCl), that readily reacts with primary and secondary

Abbreviations: GC, gas chromatography; MS, mass spectrometry; NICI, negative ion chemical ionization; PBB, *o*-(pentafluorobenzyloxycarbonyl)-benzoyl; PBTFB, *o*-(pentafluorobenzyloxycarbonyl)-2,3,4,5-tetrafluorobenzoyl; MPH, methylphenidate; IS, internal standard; ADHD, attention-deficit hyperactive disorder; LOQ, limit of quantification; HFB, heptafluorobutyryl.

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amines and under special conditions with aliphatic and phenolic hydroxyls [24]. The obtained derivatives display the beneficial characteristics of a pentafluorobenzyl ester during NICI detection, resulting in striking sensitivity due to reduced fragmentation and high efficacy of the dissociative resonance electron capture process.

The use of stable isotope labelled analogues as internal standards comprises a major advantage of mass spectrometric detection. This standardisation method has previously been applied to the analysis of MPH [23].

For pharmacokinetic applications robustness and short analysis time is a major concern, since they involve the processing of a large a number of samples. Additionally, as the target group for MPH medication consists mainly of children, reduction of sample size is of critical importance. It was therefore the aim of this study to elaborate a method for the determination of MPH in human plasma that meets the requirements of sensitivity, selectivity, speed and ruggedness for pharmacokinetic applications and drug monitoring.

2. Materials and methods

2.1. Chemicals and reagents

Pentafluorobenzyl alcohol was purchased from ABCR (Kahrlsruhe, Germany). 2,3,4,5-Tetrafluorophtalic anhydride was supplied by Sigma-Aldrich (Vienna, Austria). Methylphenidate was from Cerilliant (USA). PBTFBCl reagent was self-synthesized, but can be obtained through BPA laboratories (Graz, Austria; www.bpa-lab.com) All other substances, solvents and reagents of analytical grade were from Merck (Darmstadt, Germany).

2.2. Synthesis of o-(pentafluorobenzyloxycarbonyl)-2,3,4,5-tetrafluorobenzoyl chloride (PBTFBCl)

Synthesis of the PBTFBCl reagent was accomplished as previously described [24]. Briefly, 2,3,4,5-tetrafluorophtalic anhydride (220 mg, 1 mmol) and pentafluorobenzyl alcohol (198 mg, 1 mmol) were allowed to react at 220 °C for 1 h. After purification, 41.8 mg of the above intermediate product were treated with 0.5 mL of thionyl chloride at room temperature for 1 h. Excess reagent was removed under nitrogen and the oily residue dissolved in 10 mL of dichloromethane, yielding a 10 mM solution of the PBTFBCl reagent.

2.3. Preparation of $[^{18}O^{2}H_3]$-MPH

MPH·HCl (10 mg) was dissolved in 800 μL H218O and 20 μL fuming HCl was added. The vial was closed under nitrogen and kept at 100 °C for 72 h. After cooling the mixture was evaporated to dryness under nitrogen. The dry residue was dissolved in 300 μL of $[^{2}H_3]$-methanolic HCl (prepared by adding 20 μL of acetyl chloride to 0.5 mL of $[^{2}H_3]$-methanol) and left for 1 h at room temperature. Solvent and reagent were removed under nitrogen and the dry residue dissolved in acetonitrile isotope distribution was checked by GC–NICI–MS.

2.4. Plasma sample preparation and derivatisation

1.9 ng of the internal Standard $[^{18}O^{2}H_3]$-MPH (50 μL of an acetonitrile working solution) (IS) were pipetted into a 5-mL glass tube and 1 mL of human plasma was added. After vortexing, 1 mL carbonate buffer (pH 9.0) was added along with 1 mL of n-hexane and 100 μL of PBTFBCl reagent solution (1 mM in dichloromethane). It is crucial to add the internal standard immediately after thawing of the samples to compensate for loss by hydrolysis. The mixture was shaken on a rotary shaker for 15 min. After short centrifugation for 10 min at 4000 rpm, the (upper) n-hexane phase was transferred to a fresh glass tube and solvent evaporated under nitrogen. The dry residue was reconstituted in 100 μL of ethyl acetate and transferred to autosampler vials. 2 μL was used for GC–NICI–MS analysis using m/z 452 and m/z 413 for detection of MPH and m/z 413 and m/z 457 for the internal standard, respectively.

2.5. Gas chromatography–mass spectrometry

An ISQ quadrupole mass spectrometer coupled to a TRACE GC Ultra (Thermo Scientific, Vienna) was used. The GC was fitted with a BPX5 fused silica capillary column (15 m × 0.25 mm i.d., SGE). The injector was operated in the splitless mode at 280 °C. Helium was used as a carrier gas at a constant flow rate of 1.5 mL/min. Initial column temperature was 160 °C for 1 min, followed by an increase of 40 °C/min to 310 °C and an isothermal hold of 4 min. The mass spectrometer transfer line was kept at 315 °C. NICI was performed with methane as a moderating gas at an electron energy of 70 eV and an emission current of 0.250 A.

2.6. Analytical method validation

Calibration graphs were established in the range of 0.006 ng/mL plasma to 12.5 ng/mL plasma with twelve calibration points in duplicates. For this purpose, blank plasma was spiked with MPH to 12.5 ng/mL followed by serial dilution with blank plasma. Standard solutions of MPH were prepared in acetonitrile and stored at −20 °C. Calibration curves were established by polynomial regression analysis (quadratic fit) weighting for $1/s^2$ (s = standard deviation of duplicates). Inter-day precision was determined at 0.350 ng/mL and 16 ng/mL concentration levels by carrying 5 identical samples at each concentration level throughout the analytical sequence and subsequent analysis. This was repeated on five consecutive days. Spiked samples were prepared from blank plasma. Intra-day precision was determined at 0.006 (LOQ), 0.012, 0.024, 0.049, 0.098, 0.195, 0350 and 16 ng/mL concentration levels by carrying 5 identical samples at each concentration level throughout the analytical sequence. Spiked samples were prepared from blank plasma. Accuracy of the methods was also tested at the abovementioned concentrations. Thus, the data from precision measurements were used to calculate the deviation of the values measured from the actual spiked values. Selectivity was tested by analyzing six different blank plasma samples. Autosampler stability was determined by analyzing a set of spiked samples at different concentrations together with the corresponding calibration curve at two different days. The samples were thereby left at ambient temperature until reanalysis. Aliquot analysis was validated by analyzing 50% aliquots (500 μL sample, 1:1 dilution). Robustness towards GC temperature programming was determined by analyzing one set of samples under different GC program settings.

For assessment of accuracy and precision at prospective analytical batch size, five replicates of spiked samples at 0.350 and 16 ng/mL concentration levels together with 200 plasma samples were extracted and chromatographed with a set of calibration standards in one single run. QC samples were analyzed once before the blank plasma samples and again after analysis of 200 plasma samples. Accuracy was measured as bias (percent deviation of the calculated versus the nominal values) and precision was expressed as coefficient of variation (%).

3. Results and discussion

3.1. NICI mass spectrometry of MPH-PBTFB derivatives

As previously described, heptafluorobutyrate derivatives of MPH show a prominent fragment ion series at m/z 409/389/369/349, resulting from sequential loss of HF from the

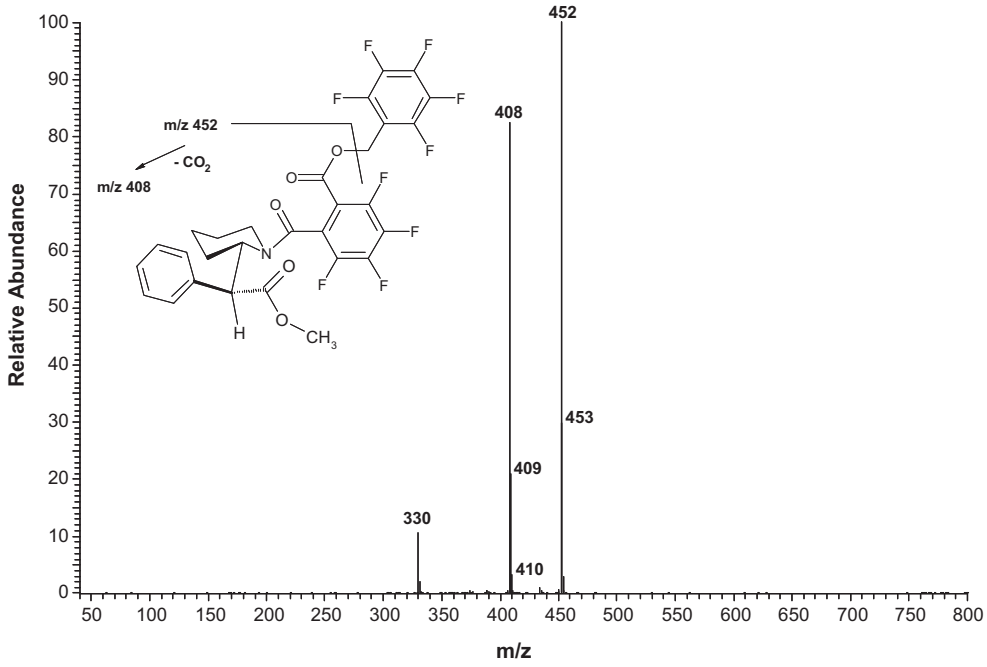

Fig. 1. NICI mass spectrum of the PBTFB derivative of methylphenidate.

molecular ion [23]. Reduced fragmentation and production of more high-mass ions by NICI, however, occurs only to a certain extent when perfluoroacyl derivatives are measured. These derivatives produce molecular anions by resonance electron capture (REC), but fragmentation is quite intense leading to the abovementioned ion series. Thus, the proportion of the total ion current is very low, no matter which fragment ion is chosen for quantification. Fragmentation under NICI conditions can, however, be drastically reduced to only a few fragment ions with striking intensity, as for pentafluorobenzyl (PFB) derivatives of carboxylic acids undergoing dissociative REC that leaves the carboxylate anion with high stability and high abundance. We have recently shown that *o*-(pentafluorobenzyloxycarbonyl)-benzoyl (PBB) derivatives also show this fragmentation mechanism and yield similar results [25]. Even better NICI response was expected for the PBTFB derivatives. The NICI mass spectrum of the PBTFB derivative of MPH is shown in Fig. 1. The carboxylate anion is present with high abundance at *m*/*z* 452, as expected. As a consequence of fluorine substitution on the aromatic core of the phthalic acid moiety, elimination reactions yield neutral loss of carbon dioxide [$M^{\bullet -}$-44] from the carboxylate anion leading to *m*/*z* 408. This is a strikingly different mass spectral behaviour as the described previously for PBB derivatives, where decarboxylation is absent and loss of ammonia occurs only to a minor extent [25]. Nevertheless, the mass spectrum still shows ions in the high mass range that all carry a large proportion of the total ion current sufficient for adequate enhancement of sensitivity. The choice between two fragment ions with high relative abundance adds the possibility to choose the *m*/*z* region of least matrix interference when applied to quantitative assays of the compound. Additionally, fluorine substitution results in higher *m*/*z* values of the used quantification masses as compared to PBB or HFB derivatives, thus shifting away from high matrix background.

3.2. Gas chromatography of MPH-PBTFB derivatives

In comparison to heptafluorobuyrate derivatives, retention times are shifted dramatically due to the bulky PBTFB group, thus allowing detection at less interference from volatile matrix components. In Fig. 2 a typical chromatogram obtained after analysis of plasma containing 0.006 ng/mL MPH is shown. As highly fluorinated compounds frequently show a carry-over effect during GC-analysis, we have performed batch analysis of samples at varying concentrations with solvent runs in between. No such carry-over could be detected for PBTFB derivatives of MPH.

3.3. Plasma sample preparation and derivatisation

The method presented here provides a rapid, rugged and simple way for the analysis of MPH in plasma allowing processing of large sample batches. Extraction and derivatisation proceeds rapidly and quantitative in this one-step procedure. The phase transfer reaction (extractive acylation) also eliminates excess reagent by hydrolysis in aqueous buffer and thus minimizes background contamination. As hydroxyls do not react under these conditions, interference from co-derivatised matrix components is low. As children are the main target of this medication, small sample size is desirable. The extraordinary sensitivity of the PBTFB derivative allows adoption of the method to use of only 250 μL of plasma and 250 μL of buffer. Amounts of reagent and hexane remain the same. Using this approach, LOQ is still very low and sufficient for most pharmacokinetic studies and drug level monitoring. If additional purification is desired, the n-hexane phase can be filtered over a short column of silica gel and eluted with ethyl acetate. This can also be used for sample concentration to avoid solvent evaporation under nitrogen, a prerequisite for possible automatisation of sample workup.

3.4. Analytical method validation

The calibration graphs established correlated well within the tested range of 0.006 ng/mL plasma to 12.5 ng/mL plasma. The regression function (quadratic fit) was $Y = 0.00357972 + 0.567072 \times X - 0.00415034 \times X^2$ ($R^2 = 0.9999$) using *m*/*z* 408 and $Y = 0.00086602 + 0.572811 \times X - 0.00440859 \times X^2$ ($R^2 = 1.0000$).

The coefficients of inter- and intra-day variation (precision) and accuracy of the spiked samples are presented in Tables 1 and 2, respectively. It can be seen from these data, that the method provides a highly precise and accurate assay for MPH in human plasma.

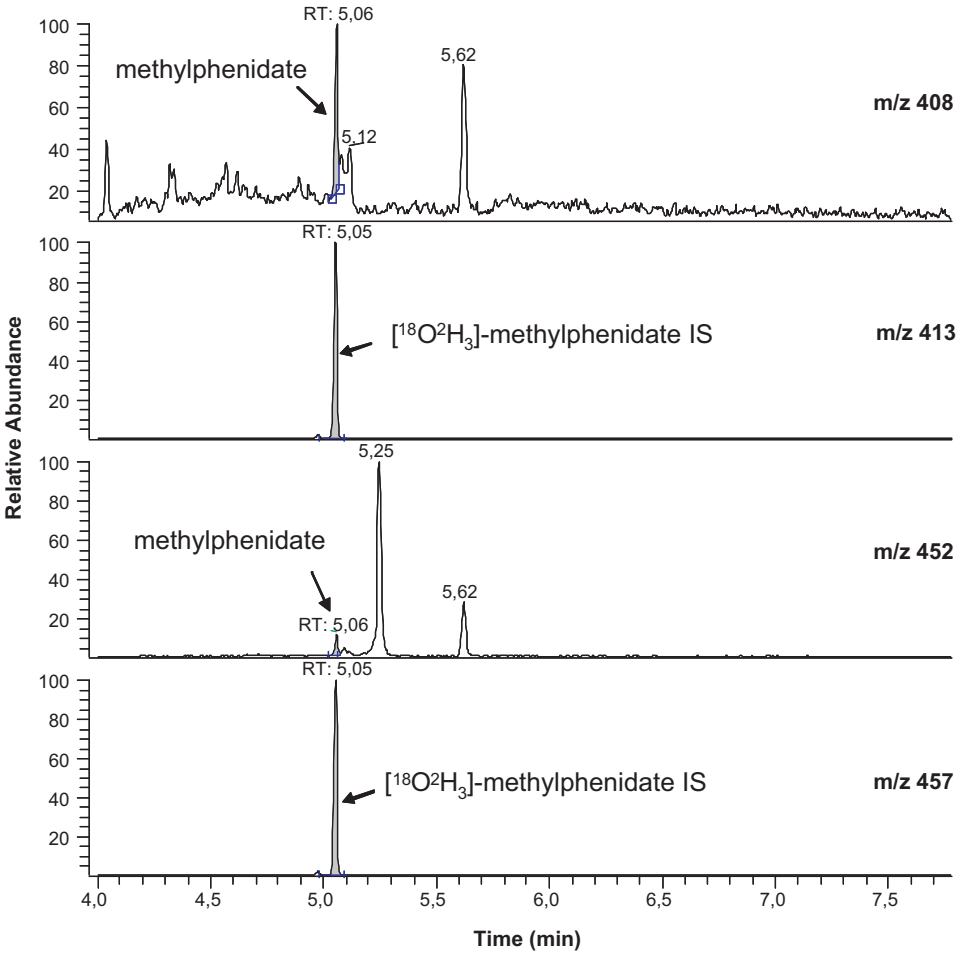

Fig. 2. Typical chromatogram obtained after analysis of plasma containing 0.006 ng/mL of methylphenidate. Samples were converted to the PBTFB derivatives and analyzed by GC–NICI–MS.

Table 1
Intra-day precision and accuracy for methylphenidate determination.

Nominal concentration (ng/mL plasma)	*m/z* 408		*m/z* 452	
	CV [%]	Accuracy [%]	CV [%]	Accuracy [%]
0.006	8.18	−4.81	4.05	19.60
0.012	2.35	−8.39	4.12	8.51
0.024	2.49	−2.51	1.21	−0.59
0.049	3.27	−3.56	4.38	−2.77
0.098	1.90	−2.77	2.00	−3.14
0.195	0.76	−1.93	2.07	−4.65
0.350	0.44	0.59	0.92	−0.20
16.000	1.79	5.16	1.29	3.46

Precision is expressed as CV (% of standard deviation from mean); accuracy is expressed as deviation (%) from nominal value.

Table 2
Inter-day precision and accuracy for methylphenidate determination.

	m/z 408		*m/z* 452	
	Nominal concentration (ng/mL plasma)			
	0.350	16.000	0.350	16.000
Mean	0.354	16.655	0.366	15.498
s.d.	0.00486	0.19741	0.00399	0.18003
CV [%]	1.38	1.19	1.09	1.16
Accuracy [%]	1.01	3.93	4.35	−3.24

Precision is expressed as CV (% of standard deviation from mean); accuracy is expressed as deviation (%) from nominal value.

For MPH, both quantification masses at *m/z* 408 and *m/z* 452 may be used with equal performance, thereby enhancing selectivity of the method. This low variability can be attributed at least in part to the use of stable isotope labelled internal standard. Mass spectrometry in combination with stable isotope dilution is a very powerful tool in external quality assessment schemes, and assays based on this technique can be regarded as reference procedures to validate other analytical methods.

Six different blank matrices were checked for interferences. In none of the samples there was background contribution above 25% LOQ.

For autosampler stability the mean concentrations of samples chromatographed immediately after sample preparation and 5 days later were measured and differed between −1.14% at 0.350 ng/mL and 2.99% at 16 ng/m (*m/z* 408) and between –0.16% at 0.350 ng/mL and 0.94% at 16 ng/m (*m/z* 452) Thus, MPH-PBTFB derivatives are stable to repeated analysis conditions.

After analyzing sample aliquots of 50% deviation was 0.91%, which shows that samples containing MPH may be measured with sufficient reliability in 500 μL sample aliquots (1:1 dilution).

Analysis of MPH with three different GC-temperature programs yielded deviations 0.80–1.1%. The method is thus robust against variations of the GC-temperature program.

Accuracy at prospective analytical batch size has been estimated for a 200 sample batch. Deviation of samples analyzed after sample batch from early analyzed samples was 4.10% at 0.350 ng/mL and 3.64% at 16 ng/mL (m/z 408), as well as −0.81% at 0.350 ng/mL

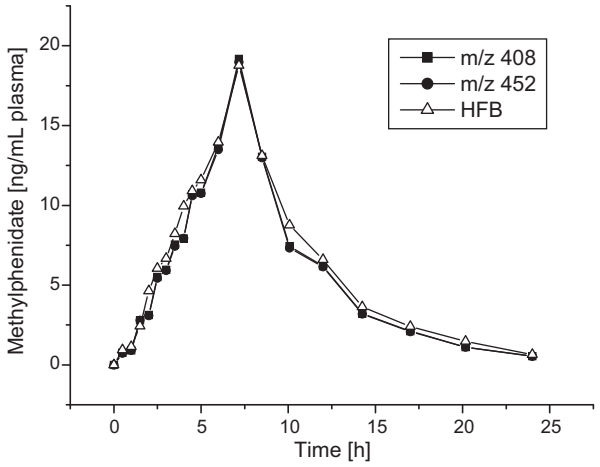

Fig. 3. Pharmacokinetic profile of methylphenidate from a human volunteer receiving 30 mg of the drug orally. (■) PBTFB derivative, *m*/*z* 408;. (●) PBTFB derivative, *m*/*z* 452;. (△) HFB derivative.

and −0.24% at 16 ng/mL (*m*/*z* 452). The method is thus suitable for analyzing batch sizes up to 200 samples.

We have applied the method described herein to the analysis of MPH during pharmacokinetic profiling of the drug. Fig. 3 shows a typical time course from a human volunteer receiving 30 mg of the drug orally. Samples were analyzed as PBTFB- and HFB derivatives, the latter being analyzed according to a previously described method [23]. The usefulness of both *m*/*z* values for quantitation, *m*/*z* 408 and *m*/*z* 452, is strongly indicated by high correlation ($R^2 = 0.99995$) of the measured values. From the pharmacokinetic profile it is clear that ultimate sensitivity down to 0.006 ng/mL is not necessarily needed for these studies. On the other hand, this offers the opportunity to use small sample aliquots with all the benefits already outlined.

Measurement of urinary levels of ritalinic acid (RA) might be an additional perspective to correlate plasma levels of MPH and its urinary metabolite. This might, to a certain extent, also be applicable or supportive in therapeutic drug monitoring, but is not suitable for pharmacokinetic profiling of the active parent drug. Additionally, when racemic MPH is administered, the inactive form is far more rapidly metabolised than the active enantiomer, thus producing the main proportion of RA in the initial phase. Distinguishing RA resulting from these enantiomers would require chiral analysis of RA and MPH in any matrix.

4. Conclusions

For the treatment of ADHD in children, an assay with highest achievable sensitivity is desirable to minimize sample size for therapeutic drug monitoring, which is crucial for adequate drug dosage because of the large intra-individual variability and tolerance. The method described fulfills these criteria exceptionally. The ease of extractive acylation keeps sample preparation time to a minimum and allows large sample batches to be analyzed in a short time. The use of a stable isotope labelled internal standards adds an additional dimension of selectivity and selectivity to the mass spectrometric detection, thereby also compensating ideally for losses during sample work-up procedure and derivatisation sequence. The extraordinary sensitivity of the assay must be attributed to the new derivatisation reagent, PBTFBCl. We have successfully applied this method to the bulk analysis of plasma samples for a preliminary pharmacokinetic study, demonstrating its ability for routine measurements.

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