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A novel HPLC fluorescence method for the quantification of methylphenidate in human plasma

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Abstract

A number of analytical methods have been established to quantify methylphenidate (MPH). However, to date no HPLC methods are applicable to human pharmacokinetic studies without the use of mass spectrometry (MS) detection. We developed a sensitive and reliable HPLC-fluorescence method for the determination of MPH in human plasma using 4-(4,5-diphenyl-1H-imidazol-2-yl) benzoyl chloride (DIB-Cl) as the derivatizing agent. An established GC-MS method was adopted in this study as a comparator assay. MPH was derivatized using DIB-Cl, and separated isocratically on a C18 column using a HPLC system with fluorescence detection (λ_{ex} = 330 nm, λ_{em} = 460 nm). The lower limit of quantification was found to be 1 ng/mL. A linear calibration curve was obtained over the concentrations ranging from 1 ng/mL to 80 ng/mL (r = 0.998). The relative standard deviations of intra-day and inter-day variations were ≤9.10% and ≤7.58%, respectively. The accuracy ranged between 92.59% and 103.06%. The method was successfully applied to the pharmacokinetic study of a subject who received a single oral dose (0.3 mg/kg) of immediate-release MPH and yielded consistent results with that of the GC-MS method. This method is the first HPLC assay with non-MS detection providing sufficient reliability and sensitivity for both pre-clinical and clinical studies of MPH.

Keywords: Methylphenidate; Pharmacokinetics; HPLC; Fluorescence detection; GC-MS

1. Introduction

The psychostimulant *dl*-methylphenidate (MPH) is the most frequently employed medication in the pharmacotherapy of attention-deficit hyperactivity disorder (ADHD), the most common neurobehavioral disorder diagnosed among children [1,2]. The marked individual variability in the dose–response relationship of MPH has been well documented over nearly 50 years of clinical use. The reasons for this extensive inter-subject variability are unclear. Pharmacokinetic factors may play a role in many cases of therapeutic failure or unpredictable side effects as large differences in plasma concentrations of MPH have been reported in subjects who received similar doses [3,4]. Therefore, a reliable and easily performed analytical method of MPH in plasma is highly desirable for clinical pharmacokinetic assessments and potential therapeutic drug monitoring studies. Additionally, MPH pharmacology continues to be investigated in *in vitro* systems as well as animal models which all require accurate analytical determinations.

A number of analytical methods using mass spectrometry (MS) as the mode of detection have been reported for the determination of MPH in plasma and other tissues, and have been considered the methods of choice [5–13]. These methods are generally both reliable and sensitive, but are not always practical due to the associated costs and availability of MS instrumentation as well as the requisite expertise in operating MS systems.

An assay published in 1979 by Soldin et al. was the only method using high-performance liquid chromatography (HPLC) coupled with a UV detector for the analysis of human plasma MPH samples, in which the lower limit of quantification (LLOQ)

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was found to be above 20 ng/mL [14]. However, this method is not suitable to apply for most MPH clinical studies because the maximal concentration of MPH in human plasma (C_{max}) is frequently below 20 ng/mL after typical dosing. In the present study, we developed an HPLC-fluorescence detection (HPLC-FL) method for the determination of MPH in human plasma by derivatization with 4-(4,5-diphenyl-1H-imidazole-2-yl) benzoyl chloride (DIB-Cl). Furthermore, an established GC-MS method was employed as a comparison [15]. This method, to our knowledge, represents the first description of non-MS detection HPLC assay that meets the requirements of selectivity and sensitivity, and is applicable to studies requiring MPH determinations.

2. Materials and methods

2.1. Chemicals

dl-MPH, the internal standards d_3-*dl*-MPH (methyl labeled) and 1-methyl-3-phenylpropylamine (MPPA), and the derivatizing agent for GC-MS determinations pentafluoropropionic anhydride were all purchased from Sigma–Aldrich (St. Louis, MO). The derivatizing agent DIB-Cl utilized in the HPLC assay was obtained from Tokyo Kasei Kogyo, Co. (Tokyo, Japan). All other agents were of analytical grade and commercially available.

2.2. Plasma sample preparation

Liquid–liquid extraction was used to isolate MPH from human plasma. Briefly, to 1 mL aliquot of plasma, 50 μL of MPPA (0.5 μM) or 50 μL of d_3-MPH (0.5 μg/mL) was added as the internal standard for HPLC-FL and GC-MS analysis, respectively. The pH was adjusted to 9.0 by adding 1 mL of sodium carbonate buffer (10 mM, pH 10.5). Following the addition of 2 mL butyl chloride/acetonitrile (4:1), the samples were mixed by shaking for 10 min, then centrifuged at 2000 × *g*. The organic phase was transferred to fresh vials and evaporated to dryness under nitrogen. For GC-MS analysis, pentafluoropropionic anhydride (50 μL) was then added as the derivatizing agent. After firmly sealing with Teflon-lined caps, the vials were heated at 50 °C for 30 min, cooled to room temperature, and evaporated to dryness under a stream of nitrogen. The residue was reconstituted with heptane (50 μL) and 2 μL was injected for analysis. For HPLC-FL analysis, the extracted samples were derivatized by adding 10 μL of 10 mM sodium carbonate (pH 9.0) and 100 μL of 1 mM DIB-Cl. The mixture was vortexed briefly, and kept at room temperature for 30 min before terminating the reaction by the addition of 10 μL of concentrated ammonia solution. Ten microlitres of the resultant mixture was subjected to HPLC-FL analysis.

2.3. Instrumentation

2.3.1. GC-MS

An Agilent model 6890 GC – 5973N MS fitted with a 5% phenylmethylpolysiloxane column (HP-5MS, 30 M × 0.25 mm, 0.25 μm film, J and W Scientific, Folsom, CA) utilizing the negative ion chemical ionization mode was employed in this study. The helium carrier gas linear velocity was 50 cm/s. Methane served as the ionization buffer. The oven was held at 90 °C for 1.5 min and then ramped to 270 °C at 20 °C/min and held for 1.5 min. MPH was detected by selected ion monitoring of *m/z* 339 from pentafluoropropionyl-MPH (M-2HF). The selected ion monitored for the derivatized internal standard d_3-MPH was 342 (M-2HF). The retention times were 9.7 min and 9.6 min for MPH and d_3-MPH, respectively.

2.3.2. HPLC-FL

The HPLC system consisted of a Waters 2690 separation module (Waters, Milford, MA), a Phenomenex Luna C18 (2) 250 mm × 4.6 mm, 5 μm reversed-phase column preceded by a 4 mm × 3 mm C18 guard column (Phenomenex, Torrance, CA), and a Waters 474 scanning fluorescence detector with the excitation and emission wavelengths set at 330 nm and 460 nm, respectively. The separation was performed at room temperature using acetonitrile/water (73/27, v/v) with the flow rate of 1.0 mL/min. Total MPH and MPPA were eluted at 10.6 min and 8.7 min, respectively.

2.4. Method validation

Calibration curves were established by spiking a series of 1 mL blank human plasma with MPH to give concentrations of 1, 4, 8, 20, 40, and 80 ng/mL. The accuracy and precision of the assay were determined from the analysis of plasma samples spiked with MPH at three concentrations representing the low, medium, and high portions of the standard curves (2, 10, and 40 ng/mL). The LLOQ was determined when the accuracy was within 80–120%, and the relative standard deviation (%R.S.D.) of precision studies was within 20%. The extraction recovery of MPH (2, 10, and 40 ng/mL) and MPPA (0.025 nmol/mL) was validated by comparing the peak areas of extracted samples to that of extracted blank plasma spiked with MPH and internal standard. The stability of DIB-MPH and DIB-MPPA derivatives was determined by injecting the analytes at 0 h and 24 h after extraction and derivatization at ambient laboratory temperature.

2.5. Application for pharmacokinetics studies

A healthy female volunteer who had provided written informed consent approved by the Medical University of South Carolina's Office of Research Integrity participated in a pharmacokinetic study within our university's General Clinical Research Center. The subject received a single 17.5 mg (0.3 mg/kg) oral dose of immediate-release MPH (Ritalin®, Novartis Pharmaceuticals, Summit, NJ). Blood samples were collected immediately prior to the administration of MPH (0 h) and again post-dosing at 0.5, 1, 1.5, 2, 3, 4, 6, 8, and 10 h time points *via* intravenous catheter. Blood collection tubes (Vacutainers®, Becton Dickinson, Rutherford, NJ) containing sodium oxalate were used to minimize post-sampling MPH hydrolysis. Samples were promptly centrifuged at 4 °C for 5 min, and the plasma was immediately transferred into

polypropylene vials and stored at −70 °C until being split and analyzed by both GC-MS and HPLC-FL methods.

3. Results and discussion

3.1. Reaction mechanism

DIB-Cl, a derivative of lophine (2,4,5-triphenyl imidazole), was originally synthesized by Nakashima et al. as a fluorescence labeling reagent [16]. DIB-Cl has proven to be a useful derivatization reagent for the HPLC-FL analysis of primary and secondary amines such as amphetamine, methamphetamine, and other sympathomimetic amines as well as bisphenol A, atomoxetine, and various other compounds [17–22]. The high fluorescence quantum yield, high selectivity, and mild derivatization reaction conditions are considered to be the merits of the DIB-Cl derivatization assay. A secondary amine in MPH is the candidate for labeling *via* N-acylation by DIB-Cl. The reaction scheme shown in Fig. 1 was predicted accordingly.

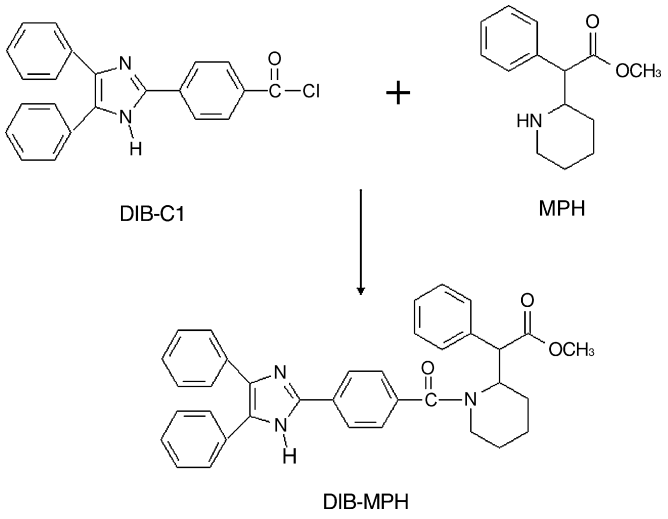

Fig. 1. The reaction scheme for labeling of MPH with DIB-Cl.

3.2. Assay validation

3.2.1. Selectivity

As shown in Fig. 2A, no endogenous components extracted from blank plasma eluted at the retention times of the peaks of either MPH or MPPA. Adequate separations were achieved at the level of LLOQ (1 ng/mL) under the current chromatographic conditions (Fig. 2B–D). The developed method was therefore found to be selective for MPH in human plasma without interferences from normal endogenous plasma constituents or the predominant yet inactive MPH metabolite, ritalinic acid.

3.2.2. Linearity

The standard curves were established using blank plasma spiked with MPH at concentrations from 1 ng/mL to 80 ng/mL. This range covers the concentrations found in human plasma within 10 h after usual dosing of MPH in most cases. The developed method was linear over the tested concentrations with a correlation coefficient of $r^2 = 0.998$.

3.2.3. Lower limit of quantification

Judged by the criteria for bioanalytical method validation presented by Shah et al. in 2000 [23], the LLOQ was found to be 1 ng/mL with a %R.S.D. of precision of 5.44% and accuracy of 110.63%. With a comparison, the same LLOQ (1 ng/mL) has been obtained by the GC-MS method using negative chemical

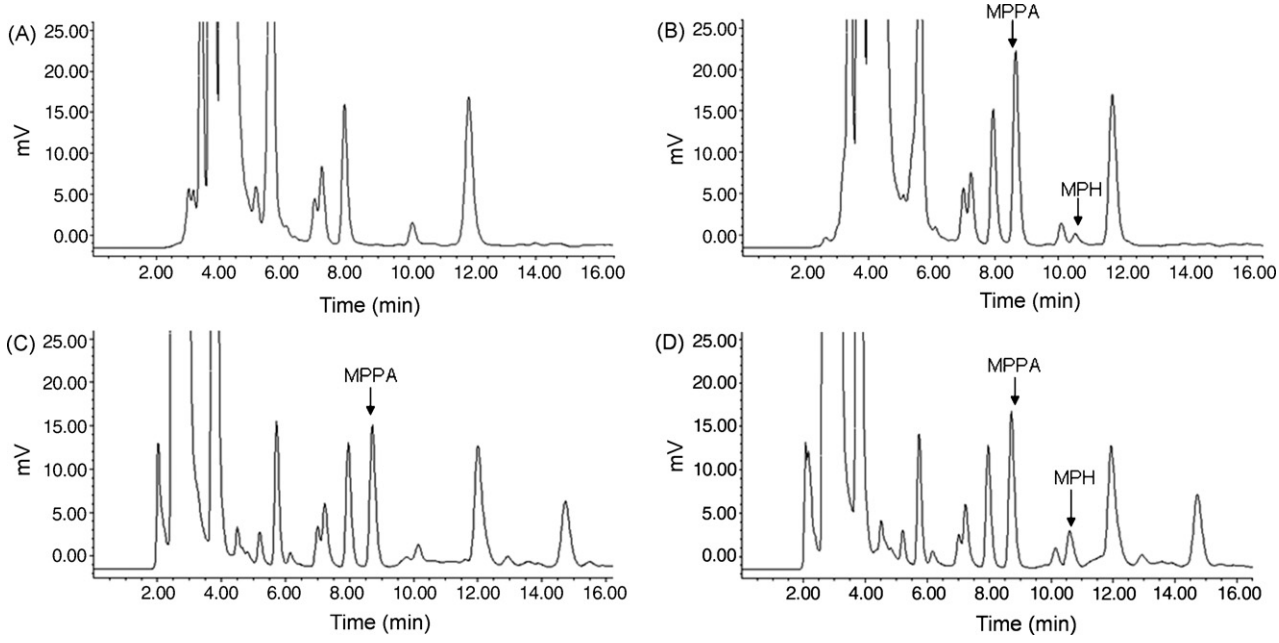

Fig. 2. Typical chromatograms of blank human plasma (A), human plasma spiked with 1 ng/mL MPH (B), plasma obtained from a healthy volunteer at the time points of 0 min (C) and 30 min (D) following a single oral dose of 0.3 mg/kg MPH.

Table 1
Intra- and inter-day precision and accuracy of GC-MS and HPLC-FL assay for the determination of MPH in human plasma

	Spiked concentration (ng/mL)					
	40		10		2	
	GC-MS	HPLC-FL	GC-MS	HPLC-FL	GC-MS	HPLC-FL
Batch 1 ($n=5$)						
Observed intra-day mean (ng/mL)	38.60 ± 0.17	40.50 ± 2.26	9.69 ± 0.36	9.71 ± 0.61	2.19 ± 0.07	1.85 ± 0.15
Intra-day precision (%)	0.44	5.59	3.77	6.24	3.04	8.05
Intra-day accuracy (%)	96.49	101.24	96.86	97.06	109.62	92.59
Batch 2 ($n=5$)						
Observed intra-day mean (ng/mL)	38.73 ± 0.90	39.52 ± 1.51	9.63 ± 0.21	10.31 ± 0.94	2.18 ± 0.08	1.96 ± 0.16
Intra-day precision (%)	2.32	3.83	2.15	9.10	3.79	8.07
Intra-day accuracy (%)	96.83	98.80	96.31	103.06	109.03	98.08
Batch 3 ($n=5$)						
Observed intra-day mean (ng/mL)	39.12 ± 0.97	39.06 ± 2.74	9.79 ± 0.16	9.46 ± 0.47	2.18 ± 0.06	1.91 ± 0.12
Intra-day precision (%)	2.48	7.00	1.64	4.98	2.74	6.50
Intra-day accuracy (%)	97.80	97.66	97.91	94.62	109.00	95.33
Inter-day ($n=15$)						
Observed inter-day mean (ng/mL)	38.82 ± 0.75	39.69 ± 2.15	9.70 ± 0.25	9.82 ± 0.74	2.18 ± 0.07	1.91 ± 0.14
Inter-day precision (%)	1.93	5.43	2.57	7.58	2.99	7.43
Inter-day accuracy (%)	97.04	99.23	97.03	98.25	109.21	95.33

ionization when pentafluoropropionic anhydride was used as the derivatizing agent.

3.2.4. Accuracy and precision

Three concentrations (2, 10, and 40 ng/mL) in five replicates were utilized to validate the accuracy and precision of the developed method. The results showed that the intra- and inter-day accuracy ranged between 92.59% and 103.06%, respectively. The %R.S.D. of intra- and inter-day precision was less than 9.10% (Table 1). In addition, the accuracy and precision of the GC-MS assay were also evaluated and shown in Table 1. All accuracy and precision results were within the acceptable limits. The developed HPLC-FL assay was thus found to meet generally accepted requirements of accuracy and precision over the studied concentration ranges which encompass all of those like encountered in treated patients.

3.2.5. Stability

Several previous studies have demonstrated that the stability of MPH in plasma was not problematic when the samples were processed within 0.5 h after thawing [9,10,13]. Therefore, during the development of this assay only the stability of the MPH and MPPA derivatives was determined by comparing the peak areas of 0 h and 24 h after the respective derivatization reactions. The results indicated that the ratios of the peaks of MPH (10 ng/mL) and MPPA (0.025 nmol/mL) of 24 h to that at 0 h were 1.02 ± 0.01 ($n=3$) and 0.97 ± 0.02 ($n=3$), respectively. This result indicates that the derivatives were sufficiently stable for at least 24 h at ambient temperatures.

3.2.6. Recovery

A typical liquid–liquid extraction method was adopted in this study. The recovery of MPH at the concentrations of 2, 10, and 40 ng/mL and MPPA (0.025 nmol/mL) were found to be 65.35% ± 6.79, 61.53% ± 9.20, 64.39% ± 5.92, and 71.09% ± 7.34, respectively. All concentrations were corrected for recovery by performing a standard curve in blank human plasma.

3.3. Analysis of human plasma

This newly developed HPLC-FL method was successfully applied to human plasma samples colleted at multiple time points from a healthy female volunteer who received a single oral dose of 0.3 mg/kg of racemic MPH. Additionally, an aliquot of each plasma sample was also analyzed by an established GC-MS method and assayed in parallel. The concentrations of nine time points between 0.5 h and 10 h after dosing were found to range from 2.04 ng/mL to 15.72 ng/mL (Fig. 3). When the results of GC-MS were used as the standards for comparison, the relative standard deviations of each time point of that of HPLC-FL method were found to be within 20%. No systematic bias was observed between the measurements of HPLC-FL and GC-

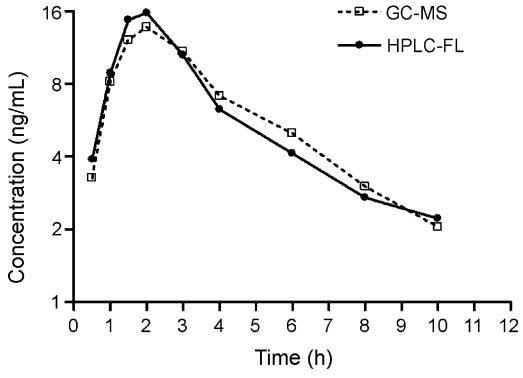

Fig. 3. Time course of plasma concentrations of MPH in a healthy volunteer receiving a single oral dose of 0.3 mg/kg MPH determined by GC-MS and HPLC-FL.

MS. Furthermore, the area under the concentration-time curves to infinity and the half-life obtained from GC-MS and HPLC-FL assays were found to be 70.59 ng h/mL, 71.66 ng h/mL, and 3.20 h, 3.23 h, respectively.

4. Conclusion

In the present study, a reliable and sensitive HPLC-FL method based on DIB-Cl derivatization for the quantification of MPH in human plasma was developed. The present assay measures total MPH concentrations and is not enantioselective. However, it is well recognized that *dl*-MPH undergoes extensive and rapid stereoselective metabolism by the carboxylesterase-1 enzyme favoring the comparatively inactive *l*-isomer [24]. As a result, only 1–5% of total circulating MPH is the *l*-MPH species, and the CNS active *d*-isomer is predominant following administration of racemic MPH [25]. Accordingly, assays for total MPH concentrations are generally viewed as adequate for most pre-clinical and clinical studies and enantiospecific assays are generally not required. Our results demonstrate that this method has excellent selectivity, linearity, precision, and accuracy, and is comparable to a previously established GC-MS assay.

This novel HPLC-FL method was successfully applied to serial plasma samples from an adult subject participating in a MPH pharmacokinetic study and compared favorably with a GC-MS method which was also performed on plasma aliquots taken from each time point sampling. The investigators recognize that the lack of data on multiple subjects represents a limitation in our assertion that this method is applicable to many clinical studies or single time point determination; however, the majority of subject samples available to us had insufficient plasma volume remaining after a previous analysis to split and compare methodologies. Nevertheless, the samples for one subject collected in a controlled environment provide additional support for our method beyond spiked plasma samples only, and some measure of comparison to alternative methods of detection.

In conclusion, the major advantage of this new assay is its simplicity and sensitivity (i.e. LLOQ of 1 ng/mL). These attributes make the assay the only HPLC method without need for detection by MS that can be applied to therapeutic drug monitoring of MPH in clinical studies, or for clinicians desiring to obtain single time point determinations from university-based or commercial laboratories. Its availability widens the ability of investigators to perform a variety of *in vitro* and *in vivo* MPH studies.

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