Development and validation of a liquid chromatography–mass spectrometry assay for hair analysis of methylphenidate

E. Marchei a, J.A. Muñoz b, Ó. García-Algar b,c, M. Pellegrini a, O. Vall b,c, P. Zuccaro a, S. Pichini a,*

a Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanità, V.le Regina Elena 299, 00161 Rome, Italy
b Pediatric Service, Hospital Universitari del Mar, Barcelona, Spain
c Unitat de Recerca Infància i Entorn (URIE), Hospital Universitari del Mar, Barcelona, Spain

Received 14 May 2007; accepted 4 June 2007
Available online 5 November 2007

Abstract

Methylphenidate (MPH) is a phenethylamine derivative used in the treatment of childhood attention-deficit hyperactivity disorder. MPH is biotransformed in the body by the hydrolysis of the methyl ester linkage to its metabolite, ritalinic acid. Whereas both compounds are usually measured in plasma and urine, preliminary observations show that only the parent compound is present in hair from treated individuals. Since in children hair samples can be easily collected without the need for specialists skills and exposing a patient to discomfort, hair testing of MPH should be an alternative to check compliance in a wider time-window than if using blood.

A procedure based on liquid chromatography–mass spectrometry (LC–MS) has been developed for the determination of MPH in hair of treated children. After addition of 3,4-methylenedioxypropylamphetamine as internal standard, hair samples were overnight digested with 0.1 M HCl at 37°C. Then, after pH adjustment to 6 using 1N NaOH, and 0.1 M phosphate buffer, the analyte was extracted with Bond–Elut Certify columns. Chromatographic separation was achieved at ambient temperature using a reverse phase column and a mobile phase of 80% 10 mM ammonium acetate–20% acetonitrile with a 20 min gradient program. The mass spectrometer was operated in positive electrospray ionization and selected ion monitoring acquisition mode.

The method was validated in the range 0.15–50 ng MPH/mg hair, using 20 mg hair per assay. At three concentrations spanning the linear dynamic range of the assay, mean recoveries ranged between 73.2 and 77.1%. First results show MPH hair concentration varying from 0.15 to 4.17 ng/mg hair, with decreasing drug concentration in distal hair segments, even in children treated with the same MPH dose during the period corresponding to different segments. This fact could be either attributed to sebum or sweat shunt with the most proximal hair segment or drug degradation by cosmetic treatments in more distal segments.

© 2007 Elsevier Ireland Ltd. All rights reserved.

Keywords: Hair; Methylphenidate; LC–MS

1. Introduction

Attention-deficit hyperactivity disorder (ADHD) is the most common neurobehavioural disorder of childhood affecting school-aged children, with a prevalence generally estimated to be 5–10% of general population [1–3]. Although multimodal treatment approaches are advocated, pharmacotherapy with psychostimulants remains a cornerstone of treatment for ADHD [2,4,5]. Of the available medications, methylphenidate (MPH), a phenethylamine derivative, is the most extensively studied and widely prescribed [6,7]. MPH is reported to be absorbed quickly and completely from the gut after oral administration [8] and it is rapidly hydrolyzed in the methyl ester linkage to its metabolite, ritalinic acid [6,8]. Minor metabolic pathways for both these compounds include parahydroxylation of the aromatic ring, oxidation to 6-oxo-derivatives and glucuronide formation [9]. Both MPH and ritalinic acid are usually measured in plasma and urine [6,8,9], but therapeutic drug monitoring for this drug is essentially lacking and early studies reported that assay of MPH blood levels did not appear to be clinically useful in the management of hyperactive children [10].
It is known that there is marked individual variability in the dose–response relationship for MPH, and therefore dosage must be titrated for optimal effect and avoidance of toxicity in each child. It is unclear whether this variability is predominantly pharmacokinetic or pharmacodynamic [11].

Monitoring exposure to therapeutic drugs in pediatric population is more difficult to perform than in adults because the need for non-invasive or less invasive, yet highly sensitive, analytical methods to assess active or passive drugs intake. This fact stimulated, in the last decades, the application of hair analysis in pediatrics [12]. Since in children hair samples can be easily collected without the need for special skills and exposing a patient to discomfort, hair testing of MPH should be an alternative to check compliance in a wider time-window than if using blood.

Several analytical methods have been reported for the determination of MPH in plasma and urine. Most of these procedures are based on ultraviolet detectors [9,13], capillary electrophoresis–mass spectrometry [14], gas chromatography–mass spectrometry [6,8,15] and liquid chromatography–tandem mass spectrometry [6]. Differently, the determination of MPH and its metabolite in hair has been never investigated. Within the framework of a study concerning the use of alternative biological matrices in therapeutic drug monitoring in pediatrics, we aimed to look for the presence of MPH and ritalinic acid in hair from treated children and eventual application of hair testing for compliance monitoring. In accordance with the fact that acidic substances are seldom incorporated in hair matrix, preliminary observations showed that ritalinic acid is absent in hair from treated children. For this reason, we focused on detection of parent drug solely in keratin matrix. In the present paper, we report the development and validation of a liquid chromatography (LC) electrospray ionization (ESI)–mass spectrometry (MS) method for determination of MPH in hair from treated children.

2. Materials and methods

2.1. Hair samples collection

Hair samples were obtained from children diagnosed for ADHD and in treatment for at least the last 6 months with different oral doses of MPH (from 5 to 36 mg/day) at Pediatric Service of Hospital del Mar, Barcelona, Spain. The study was approved by the Ethical Committee of our institution, both parents of children included in the study signed an informed consent and completed a structured questionnaire with information on treated child and MPH dosage. Hair samples (as an entire strand) were cut close to the scalp in the vertex region using a stainless steel scissors. Whereas possible (hair shaft length more than 3 cm), hair strands were divided into subsequent 3 cm segments, each representing hair growth in subsequent 3-month periods.

Drug-free human hair samples obtained from 20 non-treated children analyzed during method validation to exclude any source of chromatographic interferences and mixed to obtain a homogeneous pool of blank hair to be used for calibration standards and QC samples.

2.2. Chemicals and materials

Standard of MPH was supplied by Sigma–Aldrich (Milan, Italy) and that of 3,4-methylenedioxypropylamphetamine (MDPA used as internal standard, I.S.) by Salars (Como, Italy). Bond–Elut Certify® solid-phase extraction (SPE) columns were obtained from Varian Corp. (Harbor City, CA, USA). Ultrapure water and all other reagents of analytical grade were obtained from Carlo Erba (Milan, Italy).

2.3. Calibration standards and quality control samples

Stock standard solutions (1 mg/mL) of analytes were prepared in HPLC-grade methanol and stored at −20 °C. From stock solutions, working solutions of 10 and 1 μg/mL MPH were made and used for the preparation of calibration curves and quality control samples. I.S. was diluted in methanol to give a working solution at a concentration of 10 μg/mL and stored at −20 °C.

Calibration standards containing 0.15, 2, 5, 10, 20 and 50 ng MPH/mg hair were prepared daily for each analytical batch by adding suitable amounts of methanol working solutions to 20 mg of pre-checked drug-free hair pool sample.

Quality control (QC) samples of 0.5, 8 and 45 ng drug/mg hair and samples at the limit of quantification (LOQ) were also daily prepared in drug-free pooled hair samples. QC samples were included in each analytical batch to check calibration, accuracy and precision.

2.4. Hair sample preparation and extraction

Hair samples (20 mg) were reduced in short cuts, washed three times (2 min) with 3 mL dichloromethane in an ultrasonic water-bath and allowed to dry at room temperature.

Then, samples, calibration standards and QC samples, all added with 20 μL of I.S., were incubated in 1 mL 0.1 M HCl at 37 °C for 18 h. After digestion, samples were centrifuged at 3500 rpm for 10 min, adjusted at pH 6.0 with 100 μL 1N NaOH, diluted with 1 mL of 0.1 M phosphate buffer (pH 6.0) and applied on a Bond–Elut Certify solid-phase extraction (SPE) column, which had been preconditioned with 2 mL methanol and 2 mL 0.1 M phosphate buffer (pH 6.0).

The column was washed with 1 mL of 1.0 M acetic acid and 4 mL of methanol. The analytes were eluted with 2 mL of dichloromethane/isopropyl alcohol (80/20, v/v) with 2% ammonium hydroxide. The eluent was evaporated to dryness under a stream of nitrogen and redissolved in 100 μL of 10 mM ammonium acetate. A 20 μL volume was injected into LC column.

2.5. LC–MS analysis

LC–MS analyses were performed using an Agilent 1100 series HPLC system consisting of a G1312A binary pump, a G1322A degasser, and an ALS G1329A autosampler (Agilent Technologies, Palo Alto, CA, US) interfaced to an Agilent 1100 series G1946D mass spectrometer equipped with an atmospheric pressure ionization-electrospray (ESI) interface. Chromatographic separation was achieved using Thermo Electron-Hipersil Gold next-generation ultra pure silica column (150 mm × 4.6 mm; 5 μm) (CPS Analitica, Milan, Italy).

The mobile phase, used at a flow rate of 1 mL/min, was a gradient of a mixture of (A) 10 mM ammonium acetate and (B) acetonitrile programmed as follows: initial 80% A for 3 min, decreased to 50% A in 9 min, then increased again to 80% A in 6 min. All chromatographic solvents were degassed with helium before use. The column temperature was set at 27 °C.

The following ESI conditions were applied: drying gas (nitrogen) heated at 350 °C and used at a flow rate of 12.0 L/min; nebulizer gas (nitrogen) at a pressure of 50 psi and capillary voltage at 4000 V. Fragmentor voltage (applied to the exit end of the capillary) was 140 V, dwell time was set at 95 ms and mass peak width at 0.10 min.

The mass spectrometer was operated in positive ESI mode with selected ion monitoring (SIM) acquisition. Qualifying ions were: mz 235, 234 and 84 for MPH and mz 222, 163 and 105 for MDPA. The underlined ions were selected for quantification. The acceptance criterion for ion intensity ratios was a deviation ≤20% of the average of the ion intensity ratios of all the calibration standards.

2.6. Validation procedures

Prior to application to real samples, the method was tested in a validation protocol following the accepted criteria for bioanalytical method validation.
Selectivity, recovery, matrix effect, linearity, precision, accuracy, and limits of detection and quantification, were assayed. Selectivity tests were performed with 20 hair samples from non-treated children extracted and analyzed for assessment of potential interferences from endogenous substances. The apparent response at the retention times of MPH and I.S. were compared to the response of analyte at the LOQ and I.S. at its lowest quantifiable concentration. Furthermore, potential interferences from principal drugs of abuse such as opiates, cocaine and metabolites, cannabinoids, principal amphetamines and methylenedioxyderivatives, most used benzodiazepines, and antidepressants were also evaluated spiking 20 mg of blank hair with 1000 ng of the aforementioned substances (corresponding to 50 ng/mg hair, the highest point of calibration curve) and carried through the entire procedure.

The potential for carryover was investigated by injecting extracted blank hair pool samples, with added internal standard, immediately after analysis of the highest concentration point of the calibration curve on each of the days of the validation protocol and measuring the area of eventual peaks, present at the retention times of analyte under investigation.

Absolute analytical recoveries were calculated by comparing the peak areas obtained when QC samples were analyzed by adding the reference substance and the I.S. in the extract of drug-free hair pool samples prior to and after the extraction procedure, using four replicates for each evaluated concentration.

For an evaluation of matrix effects, the peak areas of extracted drug-free samples spiked with standards at QC concentration levels after the extraction procedure were compared to the peak areas of pure diluted substances. Calibration curves were tested over the quantification limit—50 ng MPH/mg hair. Peak area ratios between the compound and I.S. were used for calculations. A weighted (1/concentration) least-squares regression analysis was used (SPSS, version 9.0.2 for Windows). Five replicates of drug-free hair pool samples were used for calculating the limits of detection and quantification. Standard deviation (S.D.) of the mean noise level over the retention time window of MPH was used to determine the detection limit (LOD = 3 S.D.) and the quantification limit (LOQ = 10 S.D.). To be accepted, the calculated LOQ had to show precision and accuracy within the 20% relative S.D. and relative error, respectively.

Five replicates at each of three different QC sample concentrations added to drug-free hair pool samples extracted as reported above, were analyzed for the determination of intra-assay precision and accuracy. The inter-assay precision and accuracy were determined for three independent experimental assays of the aforementioned replicates. Precision was expressed as the relative S.D. (R.S.D.) of concentrations calculated for QC samples. Accuracy was expressed as the relative error of the calculated concentrations.

Mid-term stability test was performed for hair samples stored at ambient temperature. Hair pool from 10 MPH treated children was included in each analytical batch during a 3-month period. The stability was expressed as a percentage of the initial concentration (first analyzed batch) of MPH in pooled hair.

3. Results and discussion

3.1. Chromatography and validation results

A representative extracted ion chromatogram obtained following the extraction of 20 mg drug-free hair sample spiked with 40 ng MPH (2 ng/mg) and 200 ng I.S. (10 ng/mg hair) is shown in Fig. 1A. Separation of MPH and I.S. was completed in 10 min. A 8-min equilibration time was necessary at the end of each run for elution of endogenous compounds. No additional peaks due to endogenous substances that could have interfered with the detection of compound of interest were observed (Fig. 1B). Similarly, none of the drugs of abuse carried through the entire procedure, interfered with the assay. Blank samples injected after the highest point of the calibration curve did not present any traces of carryover. With respect to the matrix effect, the comparison between peak areas of MPH spiked in extracted drug-free hair samples versus those for pure diluted standard showed less than 10% analytical signal suppression due to coeluting endogenous substances.

The method exhibited good linearity along the calibration range studied. Mean calibration curves (n = 3) presented the following parameters (mean ± standard deviation): slope 0.082 ± 0.001, intercept 0.005 ± 0.001, determination coefficient ($r^2$) 0.998 ± 0.002.

Fig. 1. (A) SIM chromatograms obtained following the extraction of 2 ng/mg of MPH and 10 ng/mg I.S. spiked in drug-free hair. (B) SIM chromatograms obtained following the extraction of 20 ng drug-free hair sample. (C) SIM chromatograms obtained following the extraction of sample containing 4.17 ng/mg of MPH.
LOD and LOQ values were 0.05 and 0.15 ng/mg, respectively, and resulted adequate for the purposes of the present study. Coefficients of variation for precision and accuracy at LOQ were always better than 20%.

The absolute analytical recovery, the intra and inter-assay precision and accuracy data are presented in Table 1. Analytical recoveries were always better than 70% and showed that there were no relevant variations at different concentration levels for MPH. The results obtained for intra-assay and inter-assay precision and accuracy satisfactorily met the internationally established acceptance criteria [16].

In reference to mid-term stability test, no relevant degradation was observed in the pooled MPH hair samples analyzed in a 3-month period, with differences when compared to the initial concentration lower than 5%.

3.2. Analysis of hair samples

The method here presented has been applied to hair samples from 11 children treated with different MPH doses in a time period ranging from 6 to 24 months. Obtained results are shown in Table 2 and a chromatogram of a hair sample containing 4.17 ng MPH/mg hair is shown in Fig. 1C. MPH hair concentrations varied between 0.15 and 4.17 ng/mg hair. From these preliminary data, it seems that no linear relationship exists between the administered MPH dose and its hair concentration in subsequent hair segments from children treated with the same MPH dose during the period corresponding to different segments. Either when we consider the most proximal hair segments ($r^2 = 0.05$), which always showed higher MPH concentration, or when we consider the subsequent one, with lower concentration ($r^2 = 0.025$). With respect to the first segments, higher MPH concentration can be attributed to incorporation from sweat and sebum, as reported for other drugs [17,18]. Alternatively, lower MPH values in distal hair segments can be related to drug degradation by cosmetic treatments [19]. Nevertheless, the inter-individual variability in hair segments concentration of MPH for children treated with the same dose could be also due to variability in plasma concentrations reported for this drug [10], since drugs getting into hair have to first get into systemic circulation [20]. This latter variability, which has to be monitored in plasma samples,

Table 1
Intra-day ($n = 5$) and Inter-day ($n = 15$) precision and accuracy and recovery (mean ± S.D.) of MPH

<table>
<thead>
<tr>
<th>Analyte</th>
<th>Concentration (ng/mg)</th>
<th>Intra-assay precision (R.S.D.)</th>
<th>Intra-assay accuracy (error %)</th>
<th>Inter-assay precision (R.S.D.)</th>
<th>Inter-assay accuracy (error %)</th>
<th>Absolute recovery (%)$^a$</th>
</tr>
</thead>
<tbody>
<tr>
<td>MPH</td>
<td>0.5</td>
<td>7.7</td>
<td>13.3</td>
<td>2.8</td>
<td>13.4</td>
<td>77.1 ± 0.8</td>
</tr>
<tr>
<td></td>
<td>8</td>
<td>1.1</td>
<td>13.4</td>
<td>1.4</td>
<td>12.7</td>
<td>73.2 ± 1.7</td>
</tr>
<tr>
<td></td>
<td>45</td>
<td>6.4</td>
<td>8.1</td>
<td>1.0</td>
<td>9.5</td>
<td>75.4 ± 6.2</td>
</tr>
</tbody>
</table>

$^a$ Mean ± S.D. of four replicates.

Table 2
MPH content in hair samples from treated children

<table>
<thead>
<tr>
<th>Sample code</th>
<th>Segment (cm)</th>
<th>Administration time (months)</th>
<th>Administered dose (mg/day)</th>
<th>MPH (ng/mg hair)</th>
</tr>
</thead>
<tbody>
<tr>
<td>6A</td>
<td>0–3</td>
<td>16</td>
<td>18</td>
<td>3.74</td>
</tr>
<tr>
<td>6B</td>
<td>3–6</td>
<td></td>
<td>1.93</td>
<td></td>
</tr>
<tr>
<td>8</td>
<td>2.5</td>
<td>12</td>
<td>36</td>
<td>3.29</td>
</tr>
<tr>
<td>9A</td>
<td>0–3</td>
<td>12</td>
<td>18</td>
<td>0.84</td>
</tr>
<tr>
<td>9B</td>
<td>3–6</td>
<td></td>
<td>0.16</td>
<td></td>
</tr>
<tr>
<td>10A</td>
<td>0–3</td>
<td>12</td>
<td>30</td>
<td>1.89</td>
</tr>
<tr>
<td>10B</td>
<td>3–6</td>
<td></td>
<td>0.56</td>
<td></td>
</tr>
<tr>
<td>10C</td>
<td>6–9</td>
<td></td>
<td>0.31</td>
<td></td>
</tr>
<tr>
<td>11A</td>
<td>0–3</td>
<td>12</td>
<td>36</td>
<td>4.17</td>
</tr>
<tr>
<td>11B</td>
<td>3–6</td>
<td></td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>3</td>
<td>12</td>
<td>5</td>
<td>4.12</td>
</tr>
<tr>
<td>14A</td>
<td>0–3</td>
<td>7</td>
<td>10</td>
<td>0.48</td>
</tr>
<tr>
<td>14B</td>
<td>3–6</td>
<td></td>
<td>0.23</td>
<td></td>
</tr>
<tr>
<td>14C</td>
<td>6–9</td>
<td></td>
<td>0.15</td>
<td></td>
</tr>
<tr>
<td>15A</td>
<td>0–3</td>
<td>6</td>
<td>18</td>
<td>0.20</td>
</tr>
<tr>
<td>15B</td>
<td>3–6</td>
<td></td>
<td>0.17</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>3</td>
<td>24</td>
<td>18</td>
<td>0.67</td>
</tr>
<tr>
<td>19A</td>
<td>0–3</td>
<td>12</td>
<td>36</td>
<td>0.38</td>
</tr>
<tr>
<td>19B</td>
<td>3–6</td>
<td></td>
<td>0.21</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>1.5</td>
<td>13</td>
<td>18</td>
<td>0.18</td>
</tr>
</tbody>
</table>
problems reported with compliance during MPH treatment [21] and the common medical practice of drug discontinuation during week-ends have to be taken into proper account in the following steps of this investigation. Indeed, the number of samples analyzed in this study does not allow any statistical evaluation of obtained data. Nor the presence of MPH principal metabolite, ritalinic acid can be definitively excluded in hair samples from treated children.

In fact, although the presented methodology was initially developed and validated also for ritalinic acid, this MPH metabolite was not detected in our samples applying a LOQ of 0.2 ng/mg hair. This occurrence is in agreement with the fact that acid drugs or metabolites are seldom present in keratin matrix, which is more acidic than plasma and when it is the case substances concentration is in the range of pg/mg hair [22].

4. Conclusion

The LC–MS method reported in this paper to analyze MPH in hair, validated according to internationally accepted criteria, consists of sample digestion in hot diluted acid solution followed by solid-phase extraction, chromatographic separation from endogenous matrix components on a reversed phase column and detection in positive ESI-SIM mode. The method showed an adequate range of linearity, intra and inter-assay accuracy and precision for its application in hair analysis for assessment investigating drug-taking behaviour in treated children retrospectively over the preceding weeks or months.

An adjustment of the mass spectrometric parameters towards a better sensitivity for ritalinic acid is in development so as the analysis of a higher number of hair samples from MPH treated children to consider all the possible biological matrix and subjects variables and provide a definitive judgement on the role MPH hair testing for compliance monitoring.

References