Journal of Pharmaceutical and Biomedical Analysis 49 (2009) 434–439

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

journal homepage: www.elsevier.com/locate/jpba

Liquid chromatography–electrospray ionization mass spectrometry determination of methylphenidate and ritalinic acid in conventional and non-conventional biological matrices

Emilia Marchei[a], Magi Farrè[b], Manuela Pellegrini[a], Silvia Rossi[a], Óscar García-Algar[c], Oriol Vall[c], Simona Pichini[a,*]

[a] Department of Therapeutic Research and Medicines Evaluation, Istituto Superiore di Sanitá, V.le Regina Elena 299, 00161 Rome, Italy
[b] Bioanalysis and Analytical Services Research Group, Neuropsychopharmacology Program, Institut Municipal d'Investigació Mèdica IMIM-Hospital del Mar and Universitat Autònoma, Barcelona, Spain
[c] Paediatric Service, URIE, Hospital del Mar, and Universitat Autònoma, Barcelona, Spain

ARTICLE INFO

Article history:
Received 12 September 2008
Received in revised form
14 November 2008
Accepted 18 November 2008
Available online 27 November 2008

Keywords:
Methylphenidate
Liquid chromatography–mass spectrometry
Oral fluid
Sweat
Plasma
Urine

ABSTRACT

A procedure based on liquid chromatography–electrospray ionization mass spectrometry is described for determination of methylphenidate (MPH) and its principal metabolite ritalinic acid (RA) in plasma, urine, oral fluid and sweat using 3,4-methylendioxypropylamphetamine (MDPA) as internal standard. Aliquots of 100 μL biological fluids and sweat patch were initially treated with acetonitrile, centrifuged, and clear supernatants evaporated and redissolved in 10 mM ammonium acetate. Chromatography was performed on a reversed-phase column using a gradient of 10 mM ammonium acetate and acetonitrile as a mobile phase at a flow rate of 1 mL/min. Separated analytes were confirmed and quantified by positive electrospray ionization mass spectrometry and selected ion monitoring acquisition mode. Limits of quantifications were 1 ng/mL plasma, 1 ng/sweat patch, 0.5 ng/mL oral fluid and urine for MHF; 1 ng/mL plasma and oral fluid, 1 ng/sweat patch, 0.5 ng/mL urine for RA using 100 μL biological fluids or one sweat-patch per assay. Calibration curves were linear over the calibration ranges for both MPH and RA, with $r^2 > 0.99$. At three concentrations spanning the linear dynamic range of the assay, mean recoveries ranged between 67.9–90.3% for MPH and 36.3–92.4% for RA in the different biological matrices. This method was applied to therapeutic monitoring of MHP and RA in conventional and non-conventional biological matrices from individuals in drug treatment.

1. Introduction

Attention-deficit hyperactivity disorder (ADHD) is the most common neurobehavioral disorder of childhood affecting school-aged children, with a prevalence generally estimated to be 5–10% of general population [1–3]. At the present time methylphenidate (MPH), a psychostimulant which is a phenethylamine derivative, is the drug of choice for the treatment of ADHD [4].

MPH is reported to be absorbed quickly and completely from the gut after oral administration and it is rapidly hydrolyzed at the methyl ester linkage to its metabolite, ritalinic acid (RA) [5,6].

It is known that there is marked individual variability in the dose–response relationship for methylphenidate, and therefore dosage must be titrated for optimal effect and avoidance of toxicity in each child [7]. There are few analytical methodologies, some of them quite outdated, available for measurement of MPH and RA in plasma and urine [8–12], but therapeutic monitoring of parent drug and its metabolite is essentially lacking. Alternative biological matrices should be investigated for non-invasive assessment of short and long term drug use in paediatric and adolescent population.

Recently, we developed a procedure based on liquid chromatography–mass spectrometry (LC–MS) for the determination of MPH in hair of treated children [13]. The method has been successfully applied to monitor long-term drug compliance. Subsequently, we tried to extend the developed methodology to other conventional (blood and urine) and non-conventional (oral fluid and sweat) biological matrices. The aim of this study was twofold: to develop and validate a simple LC–MS method coupled with a minimum preparation of biological samples of small volumes (applicable to paediatric population) and to verify if non-conventional, less invasive biological matrices could be used for therapeutic MHP monitoring.

* Corresponding author. Fax: +39 06 49902016.
E-mail address: simona.pichini@iss.it (S. Pichini).

0731-7085/$ – see front matter © 2008 Elsevier B.V. All rights reserved.
doi:10.1016/j.jpba.2008.11.020

2. Experimental

2.1. Materials

Standards of MPH and RA were supplied by Sigma–Aldrich (Milan, Italy). 3,4-methylenedioxypropylamphetamine (MDPA) used as internal standard (IS) was from Salars (Como, Italy). HPLC grade solvents and all the other reagents of analytical grade were purchased from Mallinkrodt Baker (Milan, Italy). The PharmChek sweat patches were provided by PharmChem Laboratories (Menlo Park, CA, USA).

2.2. Biological samples

Blood, urine, oral fluid and sweat used for this study were obtained from one adolescent (13 years old, 47 kg and 157 cm height) and two young adults (21 ± 1 years old, 75 ± 2 kg, 180 ± 2 cm height) diagnosed for ADHD, in treatment with different dosages and formulations (fast and prolonged release) of methylphenidate attending the routine outpatient clinic at Hospital del Mar, Barcelona, Spain. The study was approved by the Ethical Committee of our institution. Parents of the adolescent and the two young adults included in this study signed an informed consent and completed a structured questionnaire. Blood, urine and oral fluid samples were collected. Blood was immediately centrifuged and the obtained plasma was pooled and aliquoted into 0.5 mL plastic tubes. Sweat patches were applied on the back of the three individuals coming for routine control, which gave their consent in applying patches before taking medication and removing them immediately after drug intake and 6 h post-application.

Drug-free plasma, urine and oral fluid were obtained from 20 different healthy donors, analysed during method validation to exclude any source of chromatographic interferences and mixed to obtain a homogeneous pool of blank samples to be used for calibration standards and quality control (QC) samples. Drug-free sweat was collected by sweat patches applied to the back of healthy donors, after the skin had been cleaned with a 70% isopropyl alcohol swab, and removed 6 h post-application. One patch from the healthy individuals was checked for the absence of principal drugs of abuse and MHP before using the other patches for calibration standards ad QC samples. All biological samples were frozen at −20 °C until analysis. No preservatives were added to specimens.

2.3. Calibration standards and quality control samples

Stock standard solutions (1 mg/mL) and working solutions (1, 0.1 and 0.01 μg/mL) of MPH, RA were prepared in methanol and stored at −20 °C until analysis. The IS working solution was used at a concentration of 0.1 μg/mL.

Calibration standards ranging from the limit of quantification (LOQ) to 100 ng MPH and RA per mL plasma, oral fluid, per sweat patch and from LOQ to 1000 ng MHP and from LOQ to 5000 ng RA per ml urine were prepared daily for each analytical batch by adding suitable amounts of methanol working solutions to 100 μL of pre-checked drug-free biological fluid or drug-free sweat patch. QC samples of 2, 40 and 80 ng/mL MPH and RA in plasma, oral fluid or ng/patch; 2, 150 and 800 ng MPH and 2, 400 and 3000 ng RA per mL urine and samples at the limit of quantification (LOQ), were prepared, aliquoted, and stored at −20 °C. QC samples were included in each analytical batch to check calibration, accuracy and precision, and the stability of samples under storage conditions. Blank urine containing 5000 ng/mL MHP and 10,000 and 15,000 ng/mL RA were prepared as over-the curve samples, to be tested for accuracy and precision once diluted 5 and 10 times, respectively.

2.4. Sample preparation

Frozen oral fluid, plasma, and urine samples were allowed to thaw at room temperature. Before analysis, oral fluid was centrifuged to discard the mucous part, which accumulated at the bottom. Aliquots of 100 μL oral fluid, plasma and urine, were added to 20 μL IS working solution and 200 μL of acetonitrile. After 30 s vortexing and 3 min centrifugation at $3000 \times g$, 150 μL supernatant was transferred to a clean extraction tube and evaporated to dryness under a nitrogen stream (40 °C, 5 min). Oral fluid, plasma and urine dried extracts were redissolved in 100 μl 10 mM ammonium acetate. For MPH and RA determination in sweat patch, the absorbent pad removed from the patch with clean tweezers added to 20 μL IS working solution in a clean tube was extracted with 1 mL of acetonitrile. The extract was evaporated to dryness and redissolved in 100 μl 10 mM ammonium acetate. A 20 μl aliquot of redissolved samples was injected onto HPLC column.

2.5. Liquid chromatography–electrospray ionization mass spectrometry

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, USA) interfaced to an Agilent 1100 series G1946D mass spectrometer equipped with an atmospheric pressure ionization-electrospray (ESI) interface. Chromatographic separation was achieved by a 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 within 9 min, then increased again to 80% A within 6 min. The mass spectrometer (Agilent 1100 series), was operated in positive electrospray ionization mode and selected ion monitoring (SIM) acquisition mode. The following ESI conditions were applied: drying gas (nitrogen) heated at 350 °C at a flow rate of 11.0 L/min; nebulizer gas (nitrogen) at a pressure of 40 psi; capillary voltage at 4000 V, fragmentor voltage (applied to the exit end of the capillary) at 140 V, dwell time 68 ms and mass peak width 0.10 min. Qualifying ions were: *m/z* 235, <u>234</u> and 84 for MPH, *m/z* 221, <u>220</u> and 84 for RA and *m/z* <u>222</u>, 163 and 105 for MDPA. The underlined ions were selected for quantification. The acceptance criterion for ion intensity ratios was a deviation ≤20% from 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 [14–15]. Selectivity, carryover, matrix effect, linearity, limits of detection and quantification, precision, accuracy, recovery, and stability were determined as previously reported [16–17].

3. Results and discussion

3.1. Chromatography and validation

Representative chromatograms obtained following the extraction of 1 ng MPH, 2 ng RA and 2 ng MDPA (IS) spiked in 100 μL drug-free plasma, urine, oral fluid and sweat patch are shown in Figs. 1A and 2A. Separation of MPH, RA and MDPA was completed in 10 min. A 5 min equilibration time was necessary at the end of each run for elution of possible endogenous compounds and system re-equilibration.

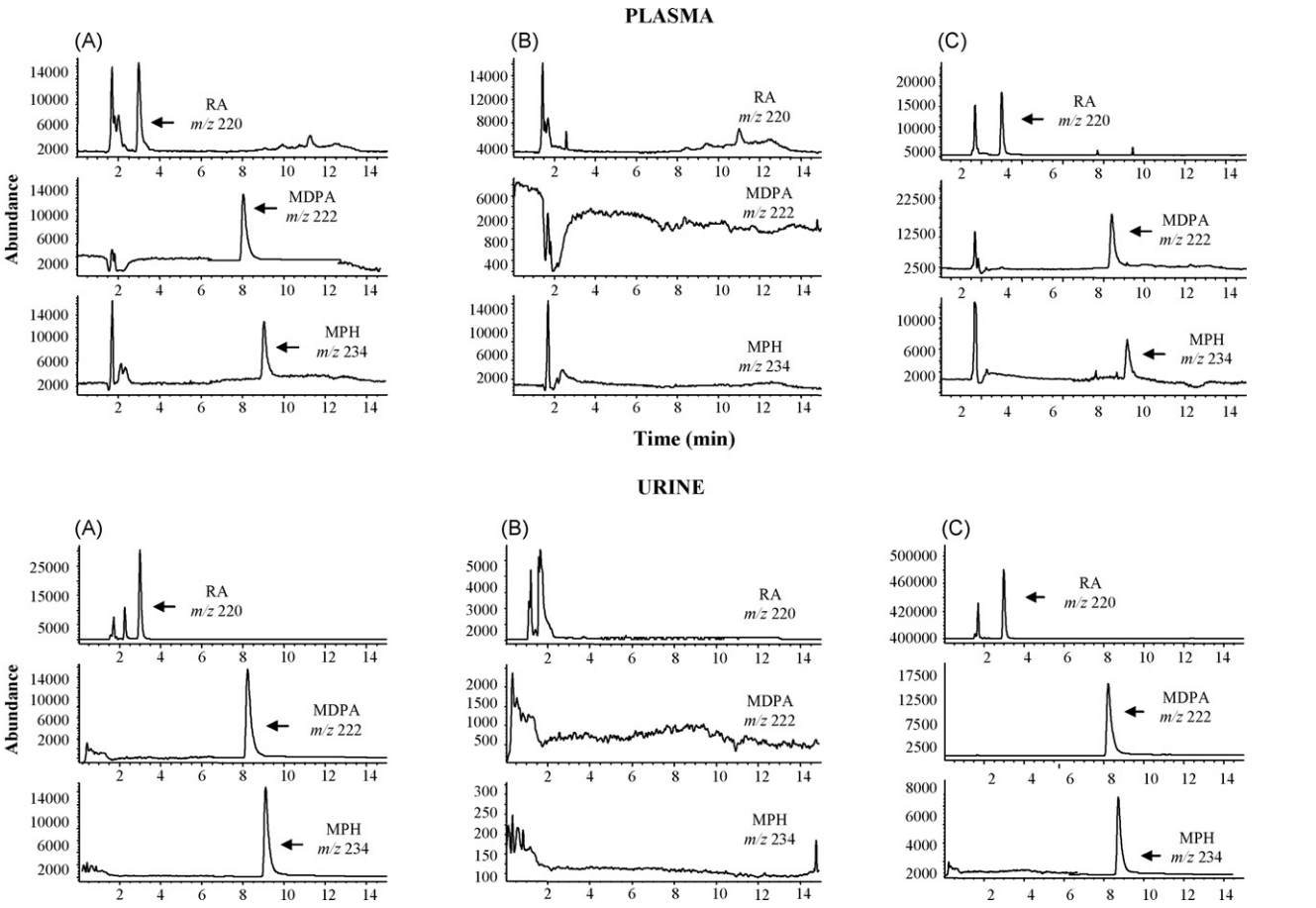

Fig. 1. SIM chromatograms of an extract of: (A) drug-free plasma (top) and urine (below) spiked with 10 ng/mL MPH, 20 ng/mL RA and 20 ng/mL MDPA; (B) drug-free plasma (top) and urine (below) samples; (C) plasma sample (top) containing 6.77 ng/mL MPH and 35.60 ng/mL RA and urine sample (below, diluted 1:10) containing 50 ng/mL MPH and 3.77 μg/mL RA.

When analyte concentration in urine samples was higher than those in the calibration curve, samples were re-processed once diluted 5 or 10 times (over-curve samples). Samples following the one exceeding the linear range in the chromatographic run were re-injected to check for possible contamination by carryover. Nevertheless, there was no carryover observed in these cases nor, when drug-free urine samples were injected after the highest point of the calibration curve.

No additional peaks due to endogenous substances that could have interfered with the detection of compounds of interest were observed after the injection of drug-free biological matrices (20 different samples per matrix) (Figs. 1B and 2B). Similarly, none of the principal drugs of abuse (opiates, cocaine, cannabinoids, amphetamines type-stimulants) or medications (antidepressants, benzodiazepines) carried through the entire procedure, interfered with the assay and with the accurate quantification of the low QC samples in all the biological matrices.

No significant ion suppression due to matrix effect (less than 10% analytical signal suppression) occurred during chromatographic runs.

Table 1
Calibration method for MPH and RA in different biological matrices.

Analyte	Calibration line slope[a]	Calibration line intercept[a]	Correlation coefficient (r^2)	Limit of detection (LOD)[b]	Limit of quantification (LOQ)[b]
Plasma					
MPH	0.041 ± 0.005	0.031 ± 0.004	0.992 ± 0.001	0.31	1.0
RA	0.109 ± 0.040	−0.107 ± 0.011	0.993 ± 0.002	0.31	1.0
Oral fluid					
MPH	0.039 ± 0.010	−0.017 ± 0.002	0.994 ± 0.003	0.15	0.5
RA	0.002 ± 0.0002	0.003 ± 0.0002	0.996 ± 0.001	0.33	1.0
Urine					
MPH	0.110 ± 0.010	0.022 ± 0.005	0.994 ± 0.003	0.14	0.5
RA	0.062 ± 0.003	0.011 ± 0.003	0.995 ± 0.004	0.16	0.5
Sweat					
MPH	0.112 ± 0.019	−0.013 ± 0.001	0.993 ± 0.001	0.30	1.0
RA	0.043 ± 0.003	−0.010 ± 0.002	0.994 ± 0.003	0.32	1.0

[a] Mean and S.D. of three replicates.
[b] ng/mL for plasma, oral fluid and urine and ng/sweat patch for sweat.

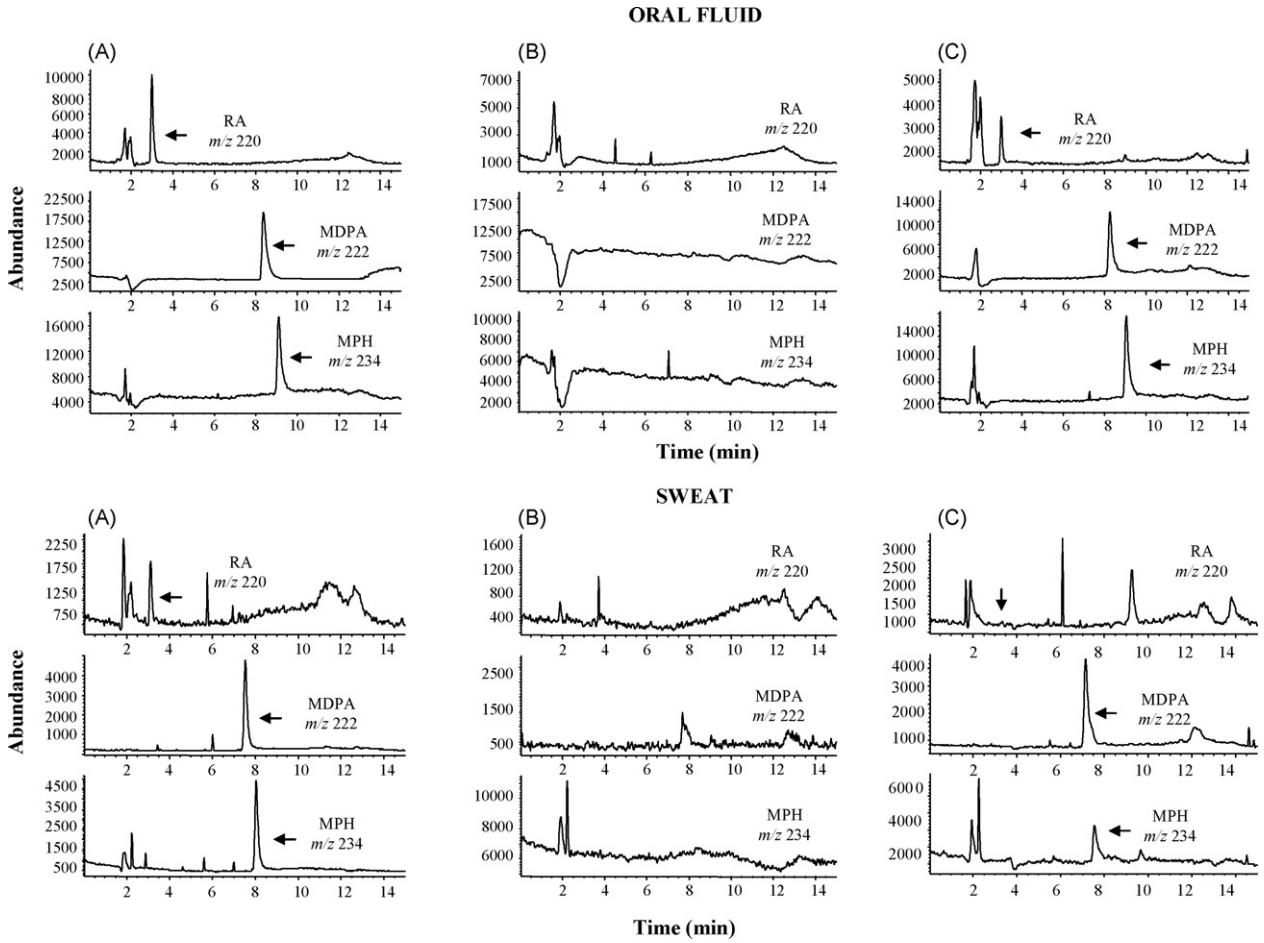

Fig. 2. SIM chromatograms of an extracts of: (A) drug-free oral fluid (top) and sweat (below) spiked with 10 ng/mL or ng/patch MPH, 20 ng/mL or ng/patch RA and 20 ng/mL or ng/patch MDPA; (B) oral fluid-free plasma (top) and sweat (below) samples; (C) oral fluid sample (top) containing 9.06 ng/mL MPH and 4.92 ng/mL RA and sweat sample (below) containing 10.15 ng/mL MPH.

Linear calibration curves showed determination coefficients (r^2) equal to or higher than 0.99 in all cases. LODs and LOQs values, calculated from S.D. of the mean noise level over the retention time window of each analyte were adequate for the purpose of the present study (Table 1). The calculated LOQ tested for precision and accuracy presented coefficients of variation always lower than 20%. The intra and inter-assay imprecision (measured as % relative standard deviation, RSD) and accuracy (measured as %error) values always lower than 20% were in accordance with the internationally established acceptance criteria [14,15] (Table 2).

Over-curve samples, tested for imprecision and accuracy after diluting 5–10 times, gave values always lower than 10% relative standard deviation (RSD) and %error. Absolute analytical recoveries (mean ± standard deviation, S.D.) obtained after extraction procedure for the three different QC samples showed that there were no significant variations at different concentration levels for the analytes under investigation in different biological matrices. The recoveries were quite high for plasma, urine and oral fluid but not for sweat patches, especially in case of RA.

Regarding the freeze/thaw stability assays for quality control samples, no significant degradation was observed after any of the three freeze/thaw cycles; the differences in concentration compared to the initial concentration were lower that 10%. Similar results (differences always lower than 10%) were obtained in case of mid-term stability test, performed re-analyzing replicates of two real plasma, urine, oral fluid samples and two sweat patches, cut in four pieces, once a month for 6 months period, assuring the validity of stored samples analysis.

3.2. Analysis of clinical samples

The method of analysis described in this report is being used for samples obtained from children and adolescents, diagnosed for ADHD, in treatment with different dosages and formulations (fast and prolonged release) of methylphenidate at Hospital del Mar, Barcelona, Spain. Our aim is to set up a therapeutic drug monitoring (TDM) to assess drug compliance, to establish the relationship between drug and metabolite concentration in different biological matrices and clinical efficacy as well as possible side-effects and finally to test the possibility of using oral fluid or sweat as non-invasive alternative biological matrices for TDM in pediatrics.

Table 3 shows the concentrations of MPH and RA in samples of different biological matrices analyzed during assay development, originating from one adolescent and two young adults selected among the subjects attending outpatient clinics at Hospital del Mar. The two adults were in treatment with 20 mg/day fast release MHP (Rubifen®, Laboratorios Rubió, Barcelona, Spain) and the adolescent with 20 mg/day prolonged release MHP (Medikinet®, MEDICE, Iserlohn, Germany). With respect to plasma samples, the peak concentrations of MPH and RA at 2 h for fast release MHP and 3 h for prolonged release MHP were similar) while at 8 h post-administration, the concentrations after administration of prolonged release drug were higher than those found in the two subjects treated with fast release formulation.

MPH appeared in oral fluid in significantly higher concentrations than those in plasma. This is not surprising due to the nature of the molecule, which is a weak base ($pK_a = 8.9$)

Table 2
Intra-assay ($n = 5$) and Inter-assay ($n = 20$) imprecision, accuracy ($n = 20$) and recovery of MPH and RA in plasma, oral fluid, urine and sweat patch.

Biological matrix and analyte	Concentration (ng/mL, and ng/patch)	Intra-assay imprecision (% RSD)	Inter-assay imprecision (% RSD)	Accuracy (% error)	Absolute recovery (%)[a]
Plasma					
MPH	2	2.4	14.0	9.1	81.8 ± 3.6
	40	3.0	7.4	12.2	77.7 ± 1.3
	80	4.4	8.4	8.5	83.3 ± 1.7
RA	2	3.0	6.2	7.5	90.2 ± 1.3
	40	8.4	4.4	5.2	92.4 ± 3.8
	80	5.4	6.4	8.2	88.2 ± 5.3
Oral fluid					
MPH	2	6.0	16.6	9.6	89.2 ± 2.1
	40	3.0	10.3	19.3	90.3 ± 1.5
	80	1.1	8.1	10.7	82.4 ± 4.8
RA	2	6.0	6.6	9.6	77.1 ± 1.2
	40	3.0	10.3	10.3	76.1 ± 0.6
	80	1.1	8.1	10.7	80.3 ± 4.7
Urine					
MPH	2	6.1	8.3	16.6	82.3 ± 5.1
	150	1.3	18.7	8.3	82.8 ± 2.9
	800	0.7	5.1	2.1	89.3 ± 3.7
RA	2	8.2	18.2	12.3	86.9 ± 2.5
	400	1.0	2.2	1.6	88.2 ± 0.3
	3000	2.1	5.8	12.6	90.4 ± 3.7
Sweat					
MPH	2	5.5	8.0	4.6	70.9 ± 4.1
	40	13.4	16.2	16.1	68.2 ± 2.5
	80	13.1	13.1	14.8	67.9 ± 3.4
RA	2	6.4	5.8	7.3	30.6 ± 5.9
	40	2.3	9.8	14.8	34.8 ± 4.5
	80	7.9	8.3	11.2	36.3 ± 1.5

and similar to other amphetamine type-stimulants (3,4-methylerndioxymethamphetamine, methamphetamine) accumulates in oral fluid which, in normal conditions (absence of salivary flow stimulation), is more acidic than blood [18]. The opposite was observed for RA, which is indeed an acidic compound and similar to other acidic substances (eg. 11-nor-Δ^9-tetrahydrocannabinol-9-carboxylic acid, THC-COOH) is not actively secreted in oral fluid [19]. In any case, parent drug and metabolite oral fluid concentration presented a great inter-subject variability, not only at peak time when a buccal contamination by tablets can be hypothesized, but also 8 h post-administration. For this reason, while these preliminary results support the analysis of MHP in oral fluid as a suitable alternative to plasma analysis in situations where monitoring in a non-invasive biological matrix is warranted, specific pharmacokinetics studies are needed to clarify if oral fluid concentrations of this drug may be a predictor of plasma concentrations.

Following 20 mg oral administration of fast release MPH in two subjects, about 50% of the administered dose was excreted in the urine in the first 8 h post-administration, primarily as RA (45.2 and 47.7%, respectively) while only 0.6 and 0.8%, respectively was excreted unchanged. Results for 8 h post-administration excretion of prolonged release formulation were slightly higher: 60% dose was excreted as RA and 3% as parent drug.

Regardless of which MPH formulation was used, only the parent drug was found in sweat in a concentration range of ten ng per sweat patch. This is in accordance with what we already

Table 3
MPH and RA concentration in samples of conventional and non-conventional matrices from three subjects following oral administration of MPH.

Subject	Drug dose (mg)	Oral fluid (ng/mL)		Plasma (ng/mL)		OF/P ratio		Amount excreted in urine in μg/mL (total amount in μg)	Sweat patch
		Peak time	8 h post-administration	Peak time	8 h post-administration	Peak time	8 h post-administration	0–8 h	8 h post-administration
1.									
MPH	20[a]	16.16	0.94	6.77	0.50	2.38	1.88	0.05 (120)	10.15
RA		1.74	2.08	35.60	14.26	0.05	0.15	3.77 (9,048)	ND[c]
2.									
MPH	20[a]	87.30	9.06	9.75	1.65	8.96	5.49	0.20 (170)	10.93
RA		6.88	4.92	39.63	15.02	0.17	0.33	11.24 (9,554)	ND[c]
3.									
MPH	20[b]	53.52	11.57	9.17	3.12	5.80	3.70	0.76 (650)	11.17
RA		18.12	12.18	29.46	18.19	0.61	0.66	14.61 (12,500)	ND[c]

[a] 20 mg Rubifen/day.
[b] 20 mg Medikinet/day.
[c] ND: not determined.

reported for amphetamine-type stimulants such as MDMA [20]. Taking into account these first observations, it can be postulated a non-negligible exctretion of MHP in sweat, which can be of use for clinical and forensic purposes.

4. Conclusion

The LC–MS method reported allows the determination of MPH and RA in conventional and non-conventional biological matrices. The rapid and simple extraction of analytes from different biological matrices, the minimum handling and time required, the high assay sensitivity and unequivocal detection render this method feasible for routine therapeutic monitoring of MPH and its metabolite RA in pharmaco-toxicological and clinical laboratories. For the first time, the possibility of drug monitoring in non-invasive biological matrices is suggested, showing that MPH and its metabolite can be detected in oral fluid, whereas only MPH is excreted in sweat, at least at clinically relevant concentrations. Hence, oral fluid can be suggested as a good non-invasive alternative to plasma for drug monitoring in pediatrics with further investigation confirming these preliminary data.

References

[1] D.P. Cantwell, J. Am. Acad. Child Adolesc. Psychiatry 35 (1996) 978–987.
[2] M. Dulcan, J. Am. Acad. Child Adolesc. Psychiatry 36 (1997) 85S–121S.
[3] J.M. Swanson, J.A. Sergeant, E. Taylor, E.J. Sonuga-Barke, P.S. Jensen, D.P. Cantwell, Lancet 351 (1998) 429–433.
[4] J.S. Markowitz, A.B. Straughn, K.S. Patrick, C.L. DeVane, L. Pestreich, J. Lee, Y. Wang, R. Muniz, Clin. Pharmacokinet. 42 (2003) 393–401.
[5] S.N. Lin, D.M. Andrenyak, D.E. Moody, R.L. Foltz, J. Anal. Toxicol. 23 (1999) 524–530.
[6] J. Eichhorst, M. Etter, J. Lepage, D.C. Lehotay, Clin. Biochem. 37 (2004) 175–183.
[7] E.J. Scharman, A.R. Erdman, D.J. Cobaugh, K.R. Olson, A.D. Woolf, E.M. Caravati, P.A. Chyka, L.L. Booze, A.S. Manoguerra, L.S. Nelson, G. Christianson, W.G. Troutman, American association of poison control centers, Clin. Toxicol. 45 (2007) 737–752.
[8] S.J. Soldin, Y.P. Chan, B.M. Hill, J.M. Swanson, Clin. Chem. 25 (1979) 401–404.
[9.M] D.L. La lande, I.J. Wilson, VcGilveray, J. Liq. Chromatogr. 10 (1987) 2257–2264.
[10] G.A. Bach, B.J. Henion, J. Chromatogr. B 707 (1998) 275–285.
[11] H.J. Leis, G. Fauler, G. Raspotnig, W. Windischhofer, J Mass Spectrom. 35 (2000) 1100–1104.
[12] M. del Mar Ramirez Fernandez, M. Laloup, M. Wood, G. De Boeck, M. Lopez-Rivadulla, P. Wallemacq, N. Samyn, J Anal Toxicol. 31 (2007) 497–504.
[13] E. Marchei, J.A. Muñoz, O. García-Algar, M. Pellegrini, O. Vall, P. Zuccaro, S. Pichini, Forensic Sci. Int. 176 (2007) 42–46.
[14] Guidance for Industry, Bioanalytical of Health and Human Services, Food and Drug Administration, May 2001. Available: http://www.fda.gov/cder/guidance/4252fnl.htm.
[15] ICH-Topic Q2B: Validation of Analytical Procedures: Methodology, International Conference on Harmonization of Technical Requirements for Registration of Pharmaceuticals for Human Use, Geneva, 1996.
[16] M. Pellegrini, E. Marchei, S. Rossi, F. Vagnarelli, A. Durgbanshi, O. García-Algar, O. Vall, S. Pichini, Rapid Commun. Mass Spectrom. 21 (2007) 2693–2703.
[17] S. Pichini, M. Pellegrini, J. Gareri, G. Koren, O. Garcia-Algar, O. Vall, F. Vagnarelli, P. Zuccaro, E. Marchei, J. Pharm. Biomed. Anal. 48 (2008) 927–933.
[18] M. Navarro, S. Pichini, M. Farré, J. Ortuño, P.N. Roset, J. Segura, R. de la Torre, Clin. Chem. 47 (2001) 1788–1795.
[19] C. Staub, J. Chromatogr. B Biomed. Sci. Appl. 733 (1999) 119–126.
[20] S. Pichini, M. Navarro, R. Pacifici, P. Zuccaro, J. Ortuño, M. Farré, P.N. Roset, J. Segura, R. de la Torre, J. Anal. Toxicol. 27 (2003) 294–303.