Journal of Pharmaceutical and Biomedical Analysis 55 (2011) 1050–1059

Contents lists available at ScienceDirect

Journal of Pharmaceutical and Biomedical Analysis

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

Determination of methylphenidate and ritalinic acid in blood, plasma and oral fluid from adolescents and adults using protein precipitation and liquid chromatography tandem mass spectrometry—A method applied on clinical and forensic investigations

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ARTICLE INFO

Article history:
Received 20 January 2011
Received in revised form 3 March 2011
Accepted 4 March 2011
Available online 11 March 2011

Keywords:
Methylphenidate
Ritalinic acid
Blood
Oral fluid
LC–MS/MS

ABSTRACT

A validated, accurate and sensitive LC–MS/MS method for determination of racemic methylphenidate and its metabolite ritalinic acid has been developed. The analytes were quantified by tandem mass spectrometry operating in positive electrospray ionization mode with multiple reaction monitoring. Blood, plasma and oral fluid samples of 100 μl were prepared by simple precipitation with 200 μl of an aqueous solution of zinc sulphate in methanol. Corresponding deuterated internal standards were used for quantification. Calibrations for methylphenidate and ritalinic acid were linear within the selected range of 0.2–30 ng/ml and 10–1500 ng/ml in blood or plasma and in the range of 1–500 ng/ml and 0.25–125 ng/ml in oral fluid, respectively. The method was successfully applied for the analysis of samples from patients treated with methylphenidate in the dose range of 36–72 mg/day and some representative ante mortem and post mortem samples from clinical and forensic toxicological investigations. A three to fourfold higher concentration of methylphenidate was found in oral fluid compared with blood while for ritalinic acid the concentrations were about 25-fold lower in oral fluid.

1. Introduction

Attention-deficit hyperactivity disorder (ADHD) is a neurobehavioral disorder treated pharmacologically with methylphenidate (MPH) in children, adolescents and adults [1,2]. MPH is a piperidine-derived molecule that contains two chiral centers and exists as four stereoisomers, i.e., the *dextro*- and *laevo*-enantiomers of *erythro*- and *threo*-methylphenidate. Most approved medications for ADHD consist of the racemic (50:50) mixture of *dl-threo*-methylphenidate [3]. The predominant pathway for metabolism of *dl-threo*-methylphenidate in human is de-esterification to the pharmacologically inactive carboxylic acid metabolite, i.e., *dl-threo*-ritalinic acid (RA) [4]. The administration of MPH can be of immediate-release (IR) and sustained-release (SR) formulations, or by the newer osmotic-release oral system (OROS) developed to reach a more substantial plasma level during the day [5]. In children, adolescents and adults there are distinct individual variations in treated dose and observed clinical efficacy [6]. One important guidance for optimal drug treatment is correlation with MPH in different matrices, i.e., therapeutic drug monitoring (TDM). Drug levels in blood or saliva can be of importance to find out the right individual dose. Especially in children and adolescents there are problems with invasive methods and therefore it is important to evaluate less invasive matrices like salivary specimen. In addition to TDM, MPH levels can be useful in compliance studies since discrepancy between verbal reports and medication adherence can be a problem [7]. More regular control of MPH levels in blood or saliva indicates when compliance is not satisfied. In a longer perspective hair analyses can be of interest.

A stimulant drug such as methylphenidate is considered to have the potential for abuse and thus of interest in forensic investigations [8].

Several non-stereospecific methods have been published for the analysis of MPH and/or RA in various human biological matrices using high performance liquid chromatography (HPLC) with fluorescence detection [9] or more sensitive and specific mass spectrometric detection, single state MS (LC–MS) [10–12] or tandem-MS (LC–MS/MS) [13–15]. However, methods for

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doi:10.1016/j.jpba.2011.03.009

co-determination of MPH and RA in humans are rare [12]. In addition to the methods mentioned above a single state LC–MS method for pharmacokinetic studies in monkey plasma [16] and a high-through LC–MS/MS method for co-determination of the parent drug and major metabolite in rat plasma has been described [17].

Enantioselective LC-methods have been developed for MPH on a macrocyclic antibiotic chiral selector (Chirobiotic-V, Vancomycin) with MS-detection [18] and RA on a protein-based stationary phase (Chiral-AGP) using UV-detection [19]. Recently an enantioselective method for MPH on a cellulose-based material (ChiralPAK AD) was used in a TDM study of children with ADHD [1].

A few multi-target LC–MS methods including MPH or RA have been published. MPH was included in a SSI-LC–MS (sonic spray ionisation) method for determination of antidepressants in human plasma [10], and RA was included in an ESI-LC–MS/MS (electro spray ionisation) of drugs of abuse in urine [15]. In both these applications solid phase extraction (SPE) sample preparation procedures were used. To the knowledge of the authors no application yet have been described for determination of MPH in post mortem blood samples from autopsy cases. Though, a growing interest of analysis in alternative matrices is seen and studies on MPH have been done in human hair [11], oral fluid (OF) and sweat samples [20,21]. Recently a single state ESI-LC–MS method for co-determination a MPH and RA in human blood, urine, sweat and OF was published [12]. The analytical procedure was based on a method previously developed for hair analysis in long-term compliance studies [11]. A simple extraction procedure with acetonitrile was used before analysis.

The aim of this work was to develop and validate a novel ESI-LC–MS/MS method with high sample throughput for determinations of MPH and RA in human blood, plasma and OF samples originating from clinical praxis as well as forensic investigations. The method outline with a selective chromatography in combination with specific tandem-MS detection fulfils the high quality standard required for accurate determinations in complex matrices like oral fluid and blood samples from human autopsy. Moreover, a co-determination of the parent drug and a major metabolite may give valuable information about inter-individual differences in metabolism. The method was aimed for controlled clinical studies as well as routine analysis at a forensic toxicology laboratory. Thus, it was applied on a limited set of samples from a pre-study for a planned clinical study as well as some representative ante mortem and post mortem samples from forensic toxicological investigations.

2. Experimental

2.1. Chemicals, reagents and reference compounds

Acetonitrile and methanol, gradient grade, methyl tert-butyl ether, HPLC grade, formic acid (98%, 25.5 M) and zinc sulphate heptahydrate (M.W. 287.54 g/mol), analytical grade, were purchased from Merck (Darmstadt, Germany). Ammonium formate, analytical-reagent grade, was purchased from Fluka (Buchs, Switzerland). Deionised water purified with a Milli-Q gradient grade water purifying system (Millipore Corporation, Bedford, MA), was used.

The reference compounds *dl-threo*-methylphenidate (MPH) HCl (M.W. 269.8 g/mol, free base 233.3 g/mol) and *dl-threo*-ritalinic acid (RA) (M.W. 219.3 g/mol) were purchased from Sigma–Aldrich (St Louis, MO, USA), methylphenidate-D9 HCl, mixture of stereoisomers (MPH-D9) (M.W. 278.8 g/mol, free base 242.3 g/mol) from CDN Isotopes (Pointe-Claire, Quebec, Canada) and ritalinic acid-D10, mixture of stereoisomers (RA-D10) (M.W. 229.3 g/mol) from TRC (North York, ON, Canada).

2.2. Solutions

Ammonium formate stock solution (1 M) was prepared by dilution of 12.6 g ammonium formate salt in 200 ml Mill-Q water. Ammonium formate mobile phase buffer (10 mM, 0.5% formic acid) was prepared by dilution of 5 ml ammonium formate stock solution and 250 μl formic acid (98%) to a final volume of 500 ml. Zinc sulphate stock (10%, w/v aq) solution was prepared by dilution of 18.9 g salt in 100 ml Mill-Q water.

Stock solutions of the reference compounds were prepared at 1.0 mg/ml in methanol. Working solutions were prepared separately for standards and controls as mixtures of MPH and RA. For blood and plasma analysis MPH/RA mixtures in methanol at 0.01/0.5, 0.1/5 and 0.5/25 μg/ml were prepared and for OF analysis at 0.1/0.025, 1/0.25 and 10/2.5 μg/ml. The internal standards were prepared as different composed mixtures of MPH-D9 and RA-D10 in methanol, for blood and plasma analysis at 0.025/0.25 μg/ml and for OF analysis at 0.25/0.0625 μg/ml. Both deuterated analogues consist of mixtures of erythro- and threo-isomers. Stock- and working solutions were stored at −20 °C.

For qualitative studies of matrix effects on ESI-MS, by post-column infusion experiments, working solutions at 0.2 μg/ml were freshly prepared for each analyte individually in methanol and ammonium formate (pH 3, 20 mM) (1:1, v/v).

2.3. Samples

Blood, plasma and OF samples were collected 6 h after morning dose from patients with ADHD, treated with a known dosage of MPH (Concerta®). The selected patients were five adolescents and five adults from an initial study for a coming clinical investigation. Patients were treated with MPH doses ranging from 36 to 72 mg/day, and samples were collected at the expected peak concentration of MPH 6 h after intake.

Blood samples were collected in Venosafe® plastic tubes containing Na-Heparin for plasma and Na-Fluoride/Na-Heparin for blood. Plasma was prepared by centrifugation 10 min at 1250 × *g*. For saliva collection, Salivette® plastic tubes containing a cotton wool swab were used. The swab was placed in the mouth for two min. After returning the swab into the Salivette® tube, the sample was centrifuged for 5 min at 2000 × *g*. All samples were divided in smaller aliquots and stored at −70 °C.

Drug free blood and plasma, collected from healthy volunteers, was purchased from the local University Hospital (Linköping, Sweden) and drug free OF was selected from volunteers from the laboratory staff.

The Ethics Committee of the Medical Faculty of Linköping University (Sweden), the Swedish Medical Products Agency and the Swedish Data Inspection Board approved the collection of patient samples. All patients gave written as well as oral informed consent before inclusion in the study.

A reference material of blood samples from forensic and clinical investigations at the National Board of Forensic Medicine (Linköping, Sweden) were analysed as well. Methylphenidate analysis was performed on special request from the investigator, physician or pathologist and was based on knowledge of prescribed medication or suspicion of misuse. Ante mortem blood samples from venal punctuation a.m. were selected from cases of therapeutic drug monitoring ($n = 7$), suspected driving under the influence of drugs ($n = 10$) and femoral post mortem blood obtained from autopsy cases ($n = 10$). In these cases neither the time for sampling nor the dose taken was known. With exception for the autopsy cases, transported at +5 °C, samples were transported to the laboratory at ambient temperature and stored at −20 °C before analysis.

2.4. Instrumentation

An electrospray liquid chromatography tandem-mass spectrometry system (ESI-LC–MS/MS) for gradient chromatography was used. The instrumentation consisted of an Acquity, Ultra Performance Liquid Chromatographic system (UHPLC), equipped with a solvent manager, a sample manager, a column manager for handling of four columns (Waters, Milford, MA) and an additional VICI 10-port valve (Valco International, Schenkon, Switzerland). Mass detection was performed on an API 4000 triple quadrupole instrument equipped with an electrospray interface (TURBOV™ source, TurbolonSpray® probe) operating in positive ion mode (Applied Biosystem/MSD Sciex, Stockholm, Sweden). The interface probe was set at 500 °C and the ion-spray needle was operated at +5000 V. Nitrogen was used as nebulizer gas (172 kPa), auxiliary gas (345 kPa), curtain gas (207 kPa), and as collision activated dissociation gas, CAD (set on 6). Instrument control, integration, and calculation were performed using Analyst™ 1.5 software. Infusion experiments for multiple reaction monitoring (MRM) optimizations and ion suppression studies were performed with a Harvard Apparatus II Plus syringe pump (Hollston, MA) at a flow rate of 10 μl/min. High-performance liquid chromatography (HPLC) was carried out on a Synergi Polar-RP 50 mm × 2 mm i.d., 2.5 μm (Phenomenex, Torrance, CA) equipped with an Opti-Solv 2 μm column inlet filter (Optimize, Portland, OR). Sample volumes of 2 μl were injected, followed by a 0.4 ml strong needle wash consisting of 2% formic acid in methanol/acetonitrile/2-propanol/water (25/25/25/25, v/v/v/v) and a 0.6 ml week needle wash consisting of formic acid (0.1 M) in methanol (50/50, v/v).

The mobile phases consisted of ammonium formate buffer (10 mM) with 0.05% formic acid for solvent A and methanol with 0.05% formic acid for solvent B. A linear gradient chromatography from 2 to 90% B-phase over 4 min was run. Total runtime including wash and reconditioning was 5 min. By using the 10-port valve in front of the MS-interface inlet the first and the last min of the mobile phase outlet were directed to waste. A flow-rate of 0.3 ml/min at 30 °C was used. Reference chromatograms are shown in Fig. 1 and the retention times in Table 1. A scheduled MRM method was prepared including the three most intense transitions for MPH, RA and the internal standards MPH-D9 and RA-D10. Transitions for other drugs of interest in ADHD treatment such as amphetamine, atomoxetine and the metabolites 4-hydroxyatomoxetine and N-desmethylatomoxetine, were included as well, Table 1. The most intense transition was used for quantification while the remaining two transitions could be used as qualifiers, Fig. 1. Matrix effects on the ESI signal during chromatography were investigated with an instrumental set-up with post-column infusion of the test analyte. In order to get a consistent and stable baseline for all transitions over the entire chromatogram, scheduled MRM was not used during these experiments. The dwell time of 20 ms was used for each transition resulting in a total MRM run cycle of 0.5 s. The extracted ion chromatograms (XICs) from each transition were investigated individually (Fig. 3).

2.5. Preparation of samples and quality controls

Into 12 mm × 75 mm glass test tubes 100 μl sample, blood, plasma or OF, 50 μl ammonium formate buffer (20 mM, pH 3) and 40 μl of IS-mixture (methanol solution) were added. For blood

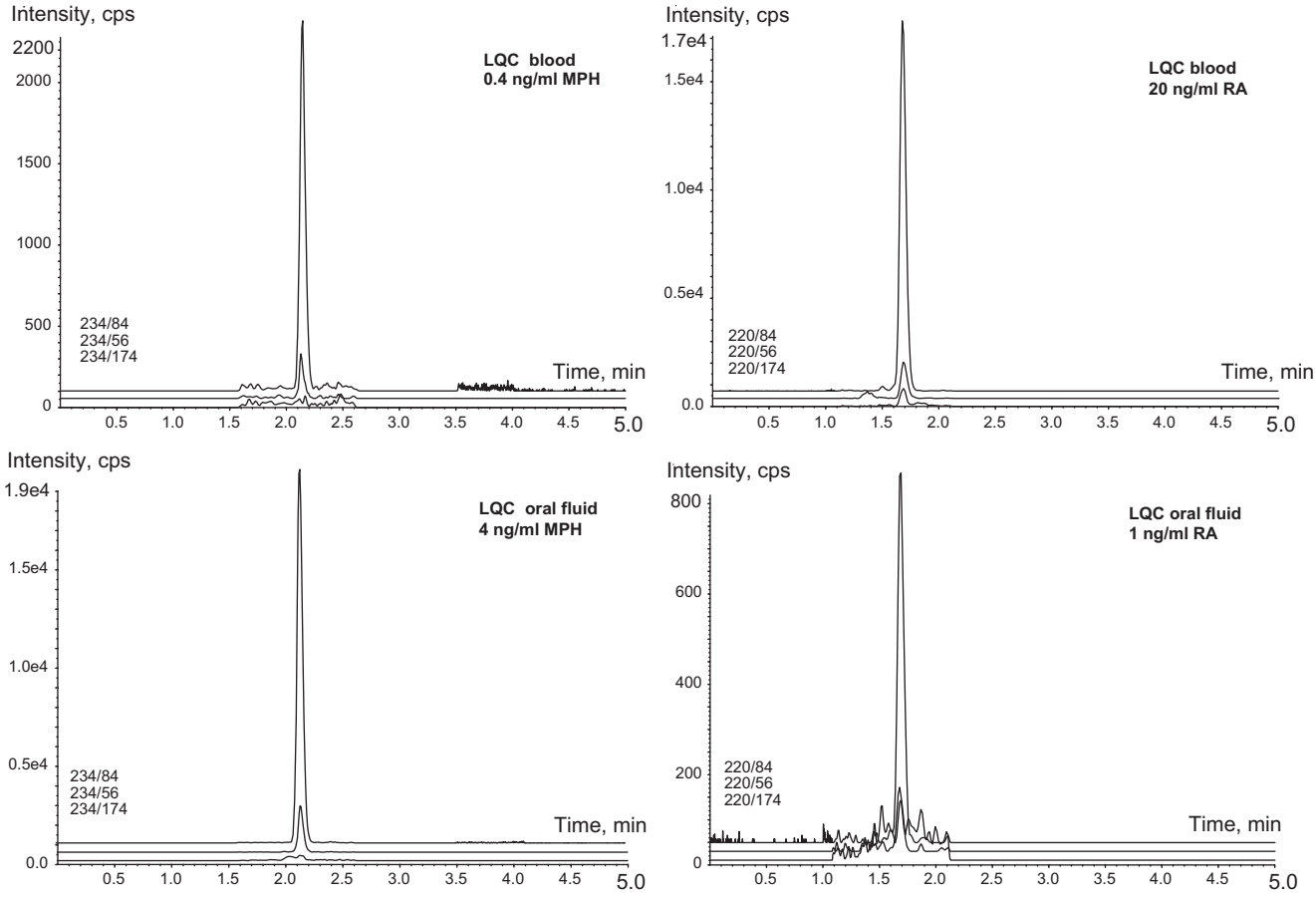

Fig. 1. LC–MS/MS chromatograms. Ion chromatograms for methylphenidate (MPH) and ritalinic acid (RA) at the low quality control level (LQC) in blood (top) and oral fluid (bottom), respectively.

Table 1
MRM transitions and retention times (Rt). Optimised declustering potential (DP), entrance potential (EP), collision energy (CE) and collision exit potential (CXP) with optimized dwell time settings by using scheduled MRM based on defined retention times.

Analyte	Transition Molecular ion	Q1/Q3 Fragment ion	DP (V)	EP (V)	CE (V)	CXP (V)	Rt (min)
MPH quantifier	234	84	65	12	30	10	2.1
MPH qualifier-1	234	56	65	12	65	8	2.1
MPH qualifier-2	234	174	65	12	30	10	2.1
RA quantifier	220	84	65	12	30	10	1.6
RA qualifier-1	220	56	65	12	65	8	1.6
RA qualifier-2	220	174	65	12	30	10	1.6
MPH-D9	243	93	65	12	30	10	2.1
RA-D10	230	93	65	12	30	10	1.6
Additional transitions							
Amphetamine quantifier	136	119	42	8	12	10	1.6
Amphetamine qualifier-1	136	91	42	8	50	10	1.6
Amphetamine qualifier-2	136	65	42	8	22	10	1.6
Amphetamine-D8	144	97	42	8	22	10	1.6
Atomoxetine quantifier	256	44	50	8	32	8	3.0
Atomoxetine qualifier-1	256	148	50	8	12	10	3.0
Atomoxetine qualifier-2	256	239	50	8	12	10	3.0
4-Hydroxyatomoxetine-1	272	44	50	8	32	8	2.0
4-Hydroxyatomoxetine-2	272	148	50	8	12	10	2.0
N-desmetylatomoxetine-1	242	30	50	8	12	10	2.5
N-desmetylatomoxetine-2	242	134	50	8	12	10	2.5

and plasma an IS-mixture consisting of 25 ng/ml MPH-D9 and 250 ng/ml RA-D10 was used, and for OF 250 ng/ml MPH-D9 and 625 ng/ml RA-D10. The samples were vortex-mixed for 10 s followed by an addition of 200 μl of precipitation solvent (5% aqueous Zinc sulphate solution (w/v) in methanol, 1:1, v/v). After 10 s vortex mixing, the test tubes were centrifuged at 4169 × *g* for 10 min. The organic phase was then transferred to 2 ml sample vials with inserts. A 2 μl aliquot was injected into the LC–MS/MS. With each batch of samples, a set of controls were prepared and analysed. To 0.5 ml blood or plasma reference compounds were added at a low concentration, LQC (0.4 ng/ml MPH, 20 ng/ml RA), at medium level, MQC (8 ng/ml MPH, 400 ng/ml RA) and at high level, HQC (25 ng/ml MPH, 1250 ng/ml RA). To 1 ml OF the reference compounds were added at a low concentration, LQC (4 ng/ml MPH, 1 ng/ml RA), at medium level, MQC (40 ng/ml MPH, 10 ng/ml RA) and at high level, HQC (400 ng/ml MPH, 100 ng/ml RA). The OF quality controls (1 ml) was adsorbed on Salivette® cotton swabs and then placed into the sampling devices for centrifugation at 4169 × *g* for 10 min. Controls in blood, plasma and OF were then treated as authentic samples.

For estimations of extraction recoveries from the Salivette® sampling device, spiked OF references were used. MPH and RA were added at the same concentrations as the QC samples in triplicates of 0.5 ml OF. A set of samples were adsorbed on Salivette® cotton swabs and re-desorbed by centrifugation before aliquots (100 μl) were taken for further preparation by protein precipitation. From another set of reference samples aliquots were taken directly for further sample preparation without passing by the Salivette® cotton swabs. Extraction recovery from protein precipitation was estimated by comparison with prepared reference samples (100%) where the precipitation solvent was exchanged by a water methanol mixture (1:1, v/v). Analyses were performed in triplicates.

For ion suppression studies, test samples (blood and OF) from five different individuals were analysed. Test samples were then treated as authentic samples before injection.

2.6. Calibration

For quantitation, calibration samples were prepared in human drug-free blood, plasma or OF by addition of the reference compound mixture (MPH and RA in methanol) to final concentrations of 0.2, 0.5, 2, 5, 10, 20 and 30 ng/ml MPH, 10, 25, 100, 250, 500, 1000 and 1500 ng/ml RA in blood or plasma, and 1, 2, 5, 20, 50, 200 and 500 ng/ml MPH, 0.25, 0.5, 1.25, 5, 12.5, 50 and 125 ng/ml in OF. The OF standards (1 ml) was adsorbed on Salivette® cotton swabs and then placed into the sampling devices before the OF solution were re-desorbed by centrifugation at 4169 × *g* for 10 min. Standard samples for calibration were then treated as authentic samples and control samples.

3. Results and discussion

3.1. Method outline

The objective was to create a sensitive and robust analytical method for the racemic MPH and the major metabolite RA that could be applied on different biological matrices like blood, plasma and OF. A single step sample preparation procedure was used in order to reduce time-consuming sample transfers and solvent evaporation. Protein precipitation with a solution of aqueous zinc sulphate and methanol was found to be highly effective for blood, plasma as well as OF. A traditional narrow bore HPLC (2 mm i.d.) with 2.5 μm particles was selected for chromatography in favour for an UHPLC material with sub-2 μm particles in order to avoid increased column backpressure caused by particles injected from the precipitates. On the selected material, Synergi Polar-RP, peak performance was favourable but no sub-2 μm particles were commercially available at the time for method development. Moreover, the phenyl material Polar-RP was selective for the diastereoisomers, *dl-erythro-* and *dl-threo-*, of methyl phenidate and ritalinic acid and peaks were well separated (Fig. 3). Though, no selectivity for the diastereoisomers was found on octadecyl when UHPLC was evaluated (HSS T3 C18, 150 mm × 2 mm, 1.8 μm particles, from Waters). The *threo*-isomer is the pharmacological relevant form of methyl phenidate and is available as the racemic mixture of the *d-* and *l-threo*-enantiomer in Concerta®. However, the non-chiral chromatography used in this work does not distinguish between the *d-* and *l*-enantiomers of the *erythro-* and *threo*-isomers. In previous published methods for the analysis of MPH and RA octadecyl materials were used but the lack of separation of the *dl-erythro* and *dl-threo* isomers was not discussed [11–13]. It is relevant though to have methods selective for the diastereoisomers of MPH and RA since commercially available deuterated standards often consist of mixtures of stereoisomers in various compositions. Moreover,

in this work it was found that one supplier of reference compounds offered the pharmacologically not relevant *erythro*-form as RA standard. The pharmacologically relevant *dl-threo*-isomers were quantified and area measurements of the corresponding *dl-threo*-isomers of MPH-D9 and RA-D10 was consequently used for calculations in this study although the *dl-threo*-isomer of MPH-D9 was about 50% and RA-D10 only was about 10% of the total amount (i.e., *dl-erthro*- and *dl-threo-isomers*) of the standard available (Fig. 3).

In order to achieve sensitive determinations with high specificity tandem-MS with MRM-analysis with multiple transitions ($n = 3$) for each analyte was used. The most abundant transition originating from product ions of the protonated molecular ion was used for quantification and for each analyte one additional transition was used as qualifier. Transitions for amphetamine, atomoxetine and metabolites, other drugs relevant in ADHD treatment, were included in the method as well. They were solely used for qualitative evaluation of unknown samples in forensic investigations.

Since the expected concentrations of MPH and RA acid differ significantly in blood/plasma and OF, different calibration ranges were needed for each analyte in respective matrices.

Different strategies for sampling of OF have been suggested and various devices have been developed and evaluated [22]. However, in a recent study of MPH pharmacokinetics on eight subjects saliva samples were collected directly in polypropylene tubes and no sampling device was used [23]. The Salivette® device selected for this study has previously been used in a controlled pharmacokinetic study on methamphetamine and amphetamine that both are chemical identities related to MPH [24]. Moreover, this device is easy to handle and simply consists of a centrifuge vessel with a 3–4 cm cylindrical cotton wool swab to be placed in the mouth and gently chewed for 1–2 min by the subjects. Then the swab is re-placed into the device, centrifuged and the OF fraction collected. Since no buffers and other additives were used and the cotton wool swab may act as a filter for food remains and other possible contaminants, some possible sources of interference were avoided.

3.2. Validation

3.2.1. Linearity and sensitivity

Linearity was evaluated by analysis of replicates ($n = 3$) of standards ($n = 7$) prepared in blood, plasma or OF. With few exceptions the mean value for each standard level was within 90–110% when a linear curve fit was used. Calibrations showed good linear response ($r \geq 0.995$) in the range of 0.2–30 ng/ml (0.9–130 nM) for MPH and 10–1500 ng/ml (46–6850 nM) for RA in blood and plasma, and in the range of 1–500 ng/ml (4.3–2140 nM) for MPH and 0.25–125 ng/ml (1.1–570 nM) for RA in OF. LOQ defined as; CV ≤ 15%, accuracy 80–120% and one qualifier ion present within a mean ratio of ≤20% of a reference value calculated from the standard samples, was estimated from replicates of control samples ($n = 5$). The acceptance criteria for the qualifier, not the intensity of the transition used for quantification, were found to be crucial for determination of LOQ. LOQ was 0.2 ng/ml for MPH and 5 ng/ml for RA in blood and 0.1 ng/ml for MPH and 2.5 ng/ml for RA in plasma. In OF LOQ was 0.1 ng/ml for MPH and 1 ng/ml RA (LQC level). If no qualifier ion is required for quantification an LOQ of 0.1 ng/ml can be achieved for RA as well. This can be compared with a recently published single state LC–MS method with protein precipitation, for the analysis of various biological matrices, where LOQ in plasma was 1 ng/ml for MPH and RA and in OF 0.5 ng/ml for MPH and 1 ng/ml for RA [12]. In a LC–MS/MS for MPH in plasma with liquid-liquid extraction using 96-well plate format a LOQ of 0.05 ng/mL was achieved [13]. However, in that method no qualifier ions were used to ensure identification. For further analysis standards in duplicates at seven levels were used. Instrument calibration was stable over several days and the same calibration was used for between day precision studies.

3.2.2. Precision and accuracy

Precision and accuracy measurements for MPH and RA were acquired from the analysis of three levels QC samples in blood or plasma (0.4, 8, 25 ng/ml MPH and 20, 400, 1250 ng/ml RA) and OF (4, 40 and 400 ng/ml MPH and 1, 10, 100 ng/ml RA) (Table 2). Between-day precision data were based on concentrations calculated from

Table 2
Validation data. Precision and accuracy for methylphenidate (MPH) and ritalinic acid (RA) determined for quality control (QC) samples.

Analyte	Added concentration ng/ml (nM)	Within-day ($n = 5$)			Between-day ($n = 5$)		
		Found concentration (ng/ml)	CV %	Accuracy %	Found concentration (ng/ml)	CV %	Accuracy %
Blood							
MPH	0.4 (1.7)	0.43 ± 0.02	4.9	108	0.40 ± 0.04	10	101
	8 (34)	7.18 ± 0.48	6.7	90	7.11 ± 0.48	6.8	89
	25 (107)	24.4 ± 2.1	8.6	98	23.5 ± 2.0	8.4	94
RA	20 (91)	22.7 ± 1.1	5.0	113	18.5 ± 2.4	13	93
	400 (1800)	386 ± 42	11	97	354 ± 46	13	88
	1250 (5700)	1212 ± 155	13	97	1038 ± 60	5.8	83
Plasma							
MPH	0.4 (1.7)	0.41 ± 0.02	4.9	104	0.39 ± 0.03	7.0	98
	8 (34)	7.73 ± 0.15	2.0	97	7.49 ± 0.21	2.9	94
	25 (107)	24.9 ± 0.9	3.7	100	25.0 ± 1.6	6.3	100
RA	20 (91)	19.7 ± 1.2	6.1	99	18.4 ± 1.2	6.7	92
	400 (1800)	377 ± 20	5.4	94	358 ± 21	5.8	89
	1250 (5700)	1163 ± 73	6.2	93	1101 ± 36	3.3	88
Oral fluid							
MPH	4 (17)	4.11 ± 0.22	5.5	103	4.5 ± 0.39	8.9	112
	40 (170)	47.4 ± 1.9	4.1	118	44.6 ± 2.8	6.2	111
	400 (1700)	404 ± 22	5.4	101	380 ± 22	5.8	95
RA	1 (4.6)	1.10 ± 0.06	5.4	110	1.10 ± 0.15	14	110
	10 (46)	11.2 ± 0.6	5.6	112	9.4 ± 0.9	9	94
	100 (460)	112 ± 9.4	8.4	112	88.2 ± 9.8	11	88

the calibration curves generated day one. For MPH the between-day precision (CV) was equal or better than 10% in blood, plasma and OF while the precision for RA was equal or better than 15%. A higher, but still acceptable, CV was achieved for RA which is most poorly retained in the chromatogram and may be affected by early eluting matrix components.

The accuracy was within 90–110% for most control levels in blood, plasma and OF, and with the exception of the HQC of RA in blood (83%) accuracy was within 85–115% for all control levels. The poorest accuracies were found for RA at the HQC (between-day data).

3.2.3. Recovery, matrix effect and stability

Recoveries were estimated from mean values of the analysis of triplicates at the three QC levels used compared with the analysis of a reference solution in water (i.e., 100%). For OF the total recoveries from Salivette® sampling and protein precipitation were estimated to 98% (LQC, 4 ng/ml), 77% (MQC, 40 ng/ml) and 79% (HQC 400 ng/ml) for MPH and 110% (LQC, 4 ng/ml), 87% (MQC, 40 ng/ml) and 77% (HQC 400 ng/ml) for RA.

For blood samples the recoveries from the protein precipitation were 98% (LQC, 4 ng/ml), 97% (MQC, 40 ng/ml) and 122% (HQC 400 ng/ml) for MPH and 115% (LQC, 4 ng/ml), 105% (MQC, 40 ng/ml) and 125% (HQC 400 ng/ml) for RA.

No severe matrix effects were observed at the retention time windows for MPH and RA when drug free reference samples: blood, plasma or OF, from different individuals ($n = 5$) were injected during post-column infusion of MPH and RA. Ion-suppression profiles for some representative samples are shown in Fig. 2.

No degradation of MPH and RA (at 10 ng and 30 ng/ml) was seen in blood and plasma when stored at −20 °C for 3 months and MPH and RA recoveries equal or better than 90% was achieved after five freeze/thaw procedures within 20 days. Some degradation of MPH and RA was observed in samples, blood, plasma and OF, at ambient temperature.

The time in room temperature were minimized during sample collection. Blood samples were divided in smaller aliquots end stored in −20 °C. OF were kept in +4 °C until centrifugation and then stored in −20 °C. Plasma samples were kept in room temperature about 45 min to avoid haemolysis before centrifugation and storage in −20 °C. Within 1–2 days all samples were transported to the final long-term storage at −70 °C.

MPH and RA recovered from protein precipitation were found stable for storage in the auto sampler at +10 °C for at least 3 days and for at least a week stored at −20 °C, when standards from calibration were reanalysed consecutively.

3.3. Application for authentic samples

The method was successfully applied for the analysis of human blood, plasma and OF samples from an initial pre-study for a clinical investigation (Table 3) as well as for blood samples from a variety of clinical and forensic cases (Table 4). Chromatograms from the analysis of blood and OF samples from a representative patient at a dose of 54 mg are shown in Fig. 3. No interferences in the extracted ion-chromatograms or severe ion suppression on the internal standard signal were seen when samples from five individuals free from MPH and RA were analysed.

For a limited set of samples MPH and RA were analysed in both blood and plasma, collected from the same patients ($n = 10$) 6 h after drug intake. Results indicate slightly higher concentrations in plasma with a mean plasma blood (P/B) ratio of 1.2 (0.8–1.7)

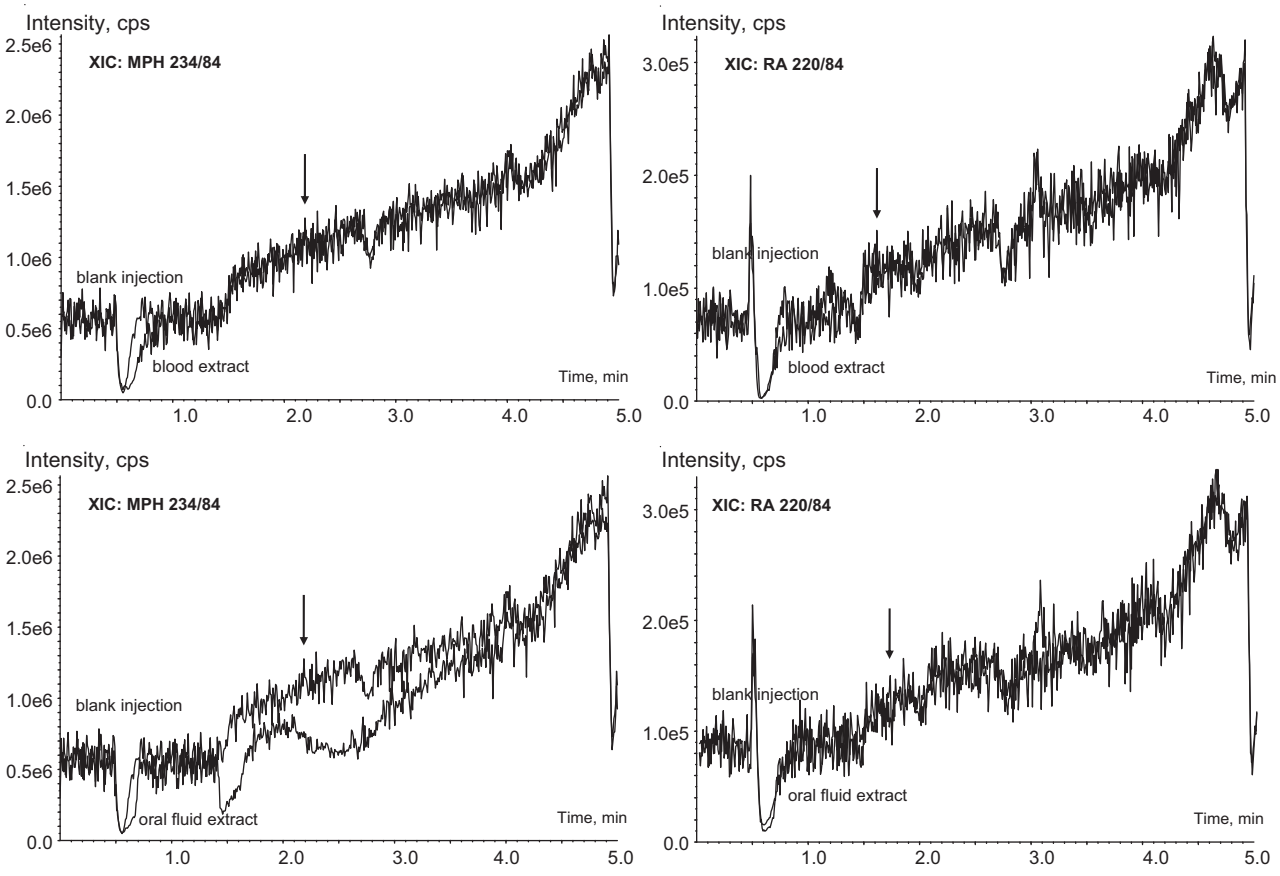

Fig. 2. Ion-suppression profiles. Ion chromatograms for methylphenidate (MPH) and ritalinic acid (RA) from injections of reference oral fluid (top) and blood samples (bottom) compared with blank injections.

Table 3
Patient data. Concentrations of methylphenidate (MPH) and ritalinic acid (RA) found in blood and oral fluid six hours after intake in ten patients treated with methylphenidate (Concerta®). Samples from a clinical pre-study.

Patient	Sex	Age	Weight	Dose	Blood					Oral fluid					Oral fluid/blood ratio	
	M/F	y	kg	mg	ng/ml	MPH nmol/mg/l	ng/ml	RA nmol/mg/l	RA/MPH Ratio	ng/ml	MPH nmol/mg/l	ng/ml	RA nmol/mg/l	RA/MPH Ratio	MPH	RA
Adolescents																
Pat. 1	M	15	90	54	13	1.1	353	30	28	48	3.8	13	1.2	0.31	3.6	0.039
Pat. 2	M	13	62	54	7.2	0.57	303	26	45	16	1.3	9.3	0.78	0.61	2.2	0.031
Pat. 3	M	17	80	54	9.2	0.73	406	34	47	29	2.3	17	1.5	0.64	3.2	0.042
Pat. 4	F	10	33	36	14	1.7	470	60	36	38	4.5	9.6	1.2	0.27	2.7	0.020
Pat. 5	M	11	33	54	22	1.7	450	38	22	88	7.0	16	1.4	0.20	4.0	0.036
Mean	**–**	**13**	**60**	**–**	**13**	**1.1**	**396**	**38**	**36**	**44**	**3.8**	**13**	**1.2**	**0.41**	**3.1**	**0.034**
Min	–	10	33	36	7.2	0.57	303	26	22	16	1.3	9.3	0.78	0.20	2.2	0.020
Max	–	17	90	54	22	1.7	470	60	47	88	7.0	17	1.5	0.64	4.0	0.042
Adults																
Pat. 6	F	19	74	54	13	0.99	450	38	37	49	3.9	18	1.5	0.39	3.9	0.040
Pat. 7	F	45	61	72	6.7	0.40	484	31	77	18	1.1	13	0.83	0.79	2.6	0.027
Pat. 8	M	18	122	54	6.2	0.50	318	27	54	22	1.8	9.6	0.81	0.46	3.6	0.030
Pat. 9	M	36	72	54	6.2	0.49	356	30	61	46	3.7	22	1.8	0.50	7.4	0.061
Pat. 10	M	24	106	36	7.5	0.89	193	24	28	36	4.2	9.4	1.2	0.28	4.8	0.049
Mean	**–**	**28**	**87**	**–**	**7.8**	**0.65**	**360**	**30**	**51**	**34**	**2.9**	**14**	**1.2**	**0.48**	**4.5**	**0.041**
Min	–	18	61	36	6.2	0.40	193	24	28	18	1.1	9.4	0.81	0.28	2.6	0.027
Max	–	45	122	72	13	0.99	484	38	77	49	4.2	22	1.8	0.79	7.4	0.061

for both MPH and RA probably due to a lower distribution of these polar compounds to the red blood cells. However, analysis of more patients is needed to prove a difference in distribution. Further on in this study only blood analysis was performed.

MPH is extensively metabolised to RA. High metabolite concentrations were found in blood 6 h after intake with RA/MPH ratio of 22–77 (Table 3). Preliminary results from these limited number of patients indicates that the RA/MPH ratio might be higher in adults compared with adolescents.

In patients from the pre-study daily oral doses extended-release MPH (Concerta®) was in the range 36–72 mg. All concentrations found were within the selected calibration ranges of the method with MPH concentrations ranging 6.2–22 ng/ml in blood and 16–88 ng/ml in OF at 6 h after intake (expected t_{max}) (Table 3). In OF an approximately three to fourfold higher concentration MPH was found compared to blood while the RA concentration was about twenty-five times lower in OF compared to blood (Table 3). The higher MPH concentration found in OF could be attributed to ion trapping of basic molecules and is in accordance with previous findings for MPH, amphetamine and methamphetamine [20,23,24]. Moreover, these findings is supported by a recent study by Marchei et al. where the correlation of MPH and RA concentration in OF and plasma were studied in three healthy subjects treated with 20 mg oral dose of extended-release MPH (Medikinet®). The MPH concentration at 2.3 h (t_{max}) was about 10-fold higher in plasma compared with OF with mean concentrations of 5.8 ng/ml in plasma and 69.5 ng/ml in OF. The opposite relation was found for RA with mean concentrations of 57.5 ng/ml in plasma and 5.6 ng/ml 2 h after administration. In contrast to data from Marchei et al. the patients in the pre-study are chronic users of MPH, which might explain the observed differences in OF/blood or plasma ratio. Moreover, drug formulation, dosage and time for sampling differ between the two studies. Further studies on inter- and intra-individual variations in OF/blood or plasma drug ratio in clinical praxis are needed.

No additional information was available, on formulation used or doses taken, for the data from forensic investigations (Table 4). However, with one exception, i.e., A10 the MPH concentrations found in blood were within the concentration range of the method and in accordance with the results from the controlled pilot study. The autopsy case A10 was a suicide by intoxication, which can explain the high MPH found. In general higher RA concentrations were found in this material compared with the samples from the controlled pre-study. A sample taken more than six hours after intake, multiple daily doses and/or a different formulation of MPH medication used, could be some explanations for these findings. For the TDM samples some information on drug treatment was available (Table 4). A comparably high RA/MPH ratio was seen in some of these cases (i.e., T01 and T02) and for some subjects the found RA concentration was above the calibration range of the method (i.e., T05, T06 and T07). However, a high given dose correlated well to a high found blood concentration in these samples. A possible explanation for these findings is that these samples originate from atypical responders under clinical investigation. In the DUID samples the RA/MPH ratio was lower than in the TDM samples and differences were less between individuals. Thus, the DUID samples may better reflect expected concentrations found during ongoing therapy. In the autopsy cases comparably low RA/MPH ratios were seen. A possible explanation for that could be that RA is less stable for autopsy samples and/or that some degradation already has occurred before sampling, transport and storage in refrigerator. Differences in MPH and RA concentrations found in blood/plasma and OF in this work supports the need for adjusted calibration ranges for analysis of various matrices.

Presented in this work is a fully validated method for MPH and RA applicable for analysis in various human matrices with a high sample throughput. By using tandem-MS detection with MRM-measurements of several transitions in combination with the use of corresponding deuterated internal standards the specificity of MPH and RA measurements in human matrices have been improved compared to previously described methods [9–12] and the risk for inaccurate measurements by the influence of matrix effects (e.g., ion suppression) have been significantly reduced. A fast chromatography within five min, selective for the *dl-erthro-* and *dl-threo*-isomers of MPH and RA, has been achieved. To the knowledge of the authors no method selective for the diastereoisomers of MPH and RA has been described elsewhere. Some preliminary results indicate that it might be relevant to be able to distinguish between the diastereomers in patient samples. Thought, the presence of the pharmacological less relevant *dl-erthro*-isomers of MPH and RA in patient samples must be further investigated. Compared to recently published methods for MPH and RA by Marchei et al. [11,12] the total runtime for analysis have been reduced about three times from 15 to 5 min. A sensitive high throughput method, within 3 min, for determination of MPH in human plasma has previously

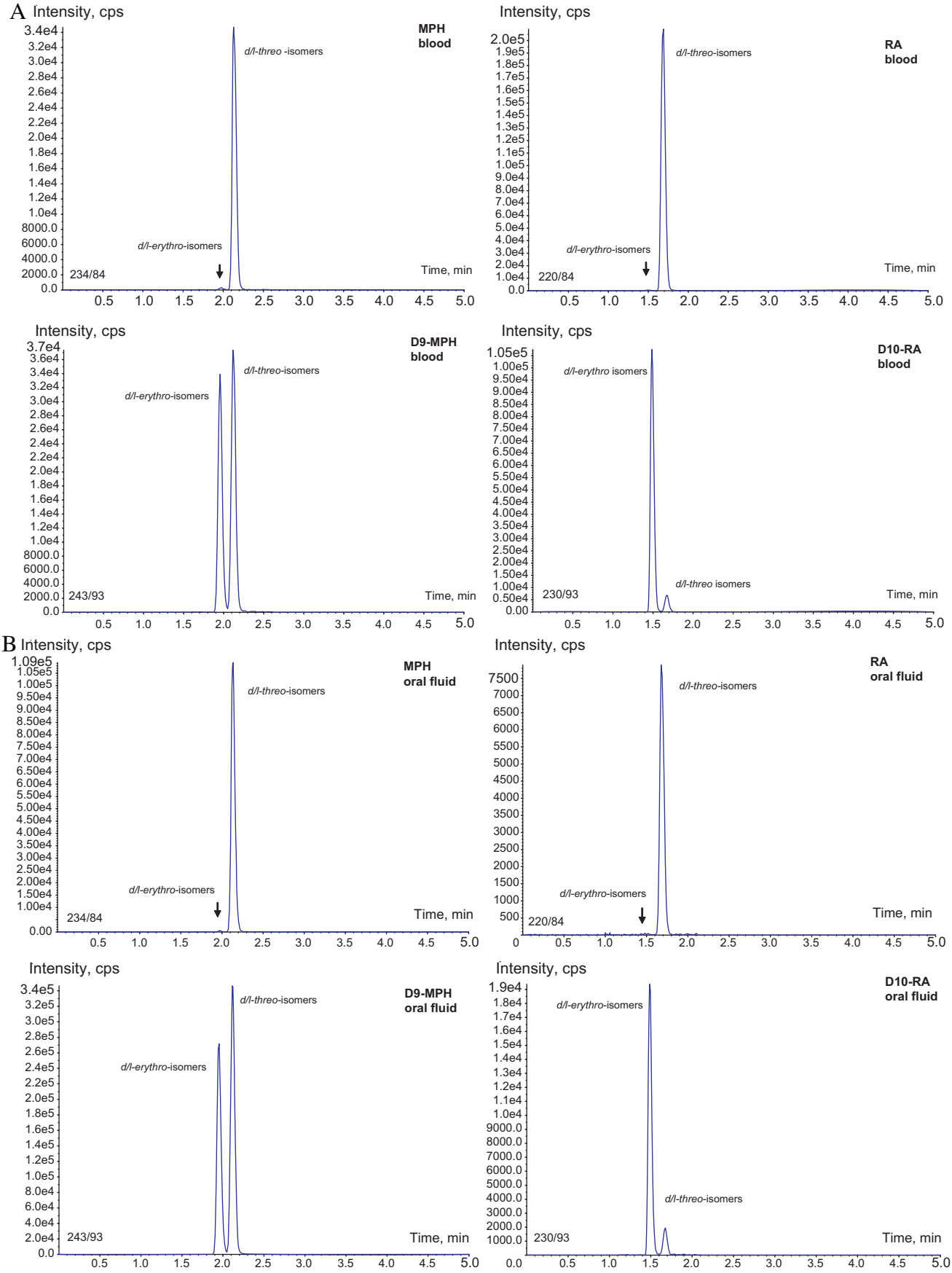

Fig. 3. Authentic sample. Ion chromatograms from an authentic sample, with 0.50 nM (6.2 ng/ml) methylphenidate (MPH) and 27 nM (318 ng/ml) ritalinic acid (RA) in blood, and 1.8 nM (22 ng/ml) MPH and 1.8 nM (9,6 ng/ml) RA in oral fluid, respectively.

Table 4
Authentic samples. Concentrations of methylphenidate (MPH) and ritalinic acid (RA) found in ante mortem blood samples from persons suspected for driving under the influence of drugs (DUID) and therapeutic drug monitoring (TDM), and post mortem femoral blood samples from toxicological investigations (autopsy).

Cases	Sex M/F	Age Years	Comments/other prescriptions or findings	Blood concentrations MPH		RA		RA/MPH
				ng/ml	nM	ng/ml	nM	Ratio
DUID								
D01	M	35	Amphetamine	Trace	–	55	251	–
D02	M	34	–	1.7	7.1	402	1836	242
D03	M	25	–	1.7	7.4	549	2507	319
D04	M	23	–	2.0	8.5	265	1208	133
D05	M	20	Paracetamol	3.8	16	251	1146	66
D06	M	28	THC	4.7	20	220	1005	47
D07	M	31	–	5.3	23	628	2868	118
D08	M	27	–	6.0	26	320	1461	53
D09	F	21	Diazepam/nordiazepam. fluoxetine	8.2	35	651	2970	80
D10	M	33	Tramadol	22	96	2080	9498	93
Mean		**28**	**Range 20–35**	**6**	**27**	**542**	**2475**	**128**
TDM			*C = capsule, T = tablets (hours since last dose)					
T01	M	35	54 mg Concerta® (18 h)/biperidene, carbamazepine, propiomazine risperidone, sertraline, zolpidem	Trace	–	60	274	–
T02	F	14	Concerta® (14 h, unknown dose)	0.1	0.3	92	420	1195
T03	M	41	Ritalin® (unknown dose)	0.4	1.7	395	1801	1009
T04	M	52	*20 mg Ritalin® C + 50 mg Ritalin® T (25 h)	1.6	6.9	164	749	102
T05	F	49	216 mg Concerta® (0.7 h)/modafinil	6.6	28	1715	7831	259
T06	F	57	*270 mg Ritalin® C (14 h)	11	49	1800	8219	159
T07	F	57	*580 mg Ritalin® C + 60 mg Ritalin® T/previous drug abuse/duloxetine, enalapril, olanzapine, sertraline, zolpidem	23	99	3900	17808	170
Mean		**44**	**Range 14–57**	**7**	**31**	**1161**	**5300**	**482**
Autopsy								
A01	M	27	Drug abuser/morphine, 6MAM, tamoxifen, THC (AAS found in urine)	Trace	–	15	68	–
A02	M	36	Drug abuser/ametamine, buprenorphine, diazepam, nordiazepam	1.7	7.5	899	4105	517
A03	M	25	Drug abuser/amphetamine (AAS found in urine)	3.2	14	230	1050	72
A04	M	27	Drug abuser/buprenorphine, norbuprenorphine, 7-amino-clonazepam	7.4	32	270	1233	37
A05	M	28	Suicide, intoxication/quetiapine	8.6	37	30	137	3
A06	F	37	Suicide, intoxication/dihydropropiomazine, hydroxizine, mirtazapine, paracetamol, venlafaxine	9.0	39	140	639	16
A07	M	49	Found dead in bed/THC	12	52	974	4445	80
A08	F	45	Suicide, hanging/citalopram, 7-amino-nitrazepam	16	70	178	811	11
A09	M	45	No comments/THC	17	73	430	1963	25
A10	M	39	Drug abuser, intoxication/carbamazepine	95	407	800	3653	8
Mean		**36**	**Range 25–49**	**19**	**81**	**397**	**1811**	**85**

been presented by Ramos et al. [13] but in this method no RA measurements were made and MPH identification was solely based on one transition.

The described method would be applied for the analysis of samples from a clinical study examining the relationship between MPH and RA concentrations in blood and OF in adult abusers, non-abusers and adolescents.

4. Conclusion

A sensitive LC–MS–MS method selective for the *dl-erythro-* and *dl-threo*-isomers of methylphenidate and ritalinic acid was developed. Determinations were successfully made in human blood, plasma as well as oral fluid samples from adolescents and adults. The method was proven to cover clinical relevant concentrations of methylphenidate and ritalinic acid, and could easily be applied for the analysis of samples from patients treated with methylphenidate according to clinical praxis as well as representative ante mortem and post mortem samples from forensic toxicological investigations. Preliminary results indicate that oral fluid may be a useful alternative matrix for the analysis of methylphenidate therapeutic measurements for within patient comparisons.

Acknowledgments

We thank Ingela Jacobsson at Linköping university hospital and Isabelle Lewung at Hospital of Oskarshamn, for skilful assistance in sample collection. The study was supported by grants from The National Board of Forensic Medicine (2007-08), FORSS (8907) and the Swedish Research Council (2009-4740).

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