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Determination of methylphenidate and its metabolite ritalinic acid in urine by liquid chromatography/tandem mass spectrometry

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ABSTRACT

Methylphenidate (MPH) is a drug that is licensed for treatment of ADHD and also narcolepsy. Monitoring of the parent drug and its major metabolite ritalinic acid (RA) in urine is considered necessary to ensure compliance with treatment programmes. A rapid, simple and sensitive liquid chromatography/tandem mass spectrometry (LC–MS/MS) assay was developed for the determination of MPH and its metabolite RA in human urine. After urine was diluted with water, methylphenidate, the major metabolite ritalinic acid, and d_6-amphetamine as the internal standard were resolved on a PFP propyl column using gradient elution of 0.02% ammonium formate and acetonitrile. The total analysis time was 13.5 min. The three compounds were detected using electrospray ionisation in the positive mode. Standard curves were linear over the concentration range 5–5000 μg/L ($r > 0.997$), bias was ≤±20%, intra- and inter-day coefficients of variation (imprecision) were <8% and the limit of detection was 5 μg/L. The limit of quantitation was set at 100 μg/L. Matrix effects were up to 140% but these were accounted for by the internal standard. The assay is being used successfully in clinical practice to enhance the safe and effective use of methylphenidate.

1. Introduction

Attention deficit hyperactivity disorder (ADHD) is a common neuro-behavioural disorder of childhood, thought to affect up to 10% of the general population [1,2].

Methylphenidate (MPH) is a psychostimulant drug approved primarily for the treatment of attention deficit hyperactivity disorder (ADHD) and narcolepsy [3]. It belongs to the piperidine class of compounds and increases the levels of dopamine and noradrenaline in the brain through reuptake inhibition of the monoamine transporters [3]. The main urinary metabolite is a de-esterified product, ritalinic acid (RA), which accounts for 80% of the dose and which has a half-life of about 8 h [3].

MPH has shown some benefits as a replacement therapy for individuals dependent on amphetamine-type substances and also has potential itself for abuse.

For treatment programmes, it is important to confirm compliance with MPH and that supplies of MPH are not being diverted for illicit use. Studies of pharmacy databases and treatment studies have shown that the prevalence of medication discontinuation or non-adherence is between 13.2% and 64% [4]. The clinical laboratory has an important role in being able to detect MPH and its metabolite RA in urine.

Various analytical methods have been developed for measuring MPH including immunoassay [5], HPLC with UV detection [6] and more recently liquid chromatography–electrospray ionisation mass spectrometry [7–9].

In recent years, HPLC with tandem mass spectrometric detection (LC–MS/MS) has been demonstrated to be a powerful technique for the quantitative determination of drugs and metabolites in biological fluids. This technique can provide high selectivity and simplification of both sample extraction procedures and chromatography [8,10,11].

Compared with other techniques, such as ELISA and HPLC–UV, LC–MS/MS can achieve higher specificity and sensitivity by utilising collision-induced dissociation while monitoring unique precursor to product ion transitions. There are also advantages of lower costs in comparison to commercially purchased ELISA kits that were being used in our laboratory. The aim of the present work was to develop and validate a rapid, simple, specific, sensitive, robust and reliable LC–MS/MS method for the determination of MPH and its metabolite RA in human urine, suitable for monitoring drug compliance. An additional aim was to use a small volume of urine and simple sample preparation without losing specificity and sensitivity.

2. Experimental

2.1. Materials

MPH (Fig. 1) and d_6-amphetamine (Fig. 1) were purchased from Cerillant (Texas, USA) as 1 mg/mL solutions in methanol and RA

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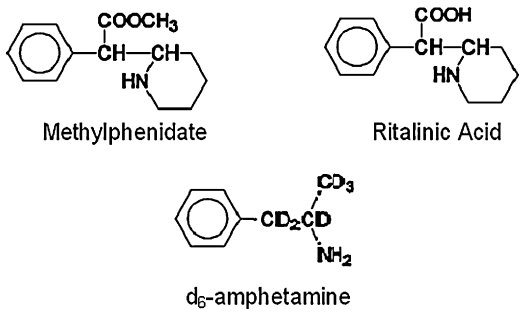

Fig. 1. Chemical structures of MPH, RA and d_6-amphetamine.

(Fig. 1) from Sigma–Aldrich (Australia). HPLC grade acetonitrile was purchased from Thermo Fisher (Cambridge, UK), ammonium formate from Sigma–Aldrich (Germany), and HPLC grade methanol and reagent grade 89–91% pure formic acid from BDH (Poole, UK). Distilled, deionised water was produced by a Milli-Q Reagent Water System (Millipore, MA, USA).

2.2. Instrumentation and analytical conditions

The LC–MS/MS system consisted of an Agilent 1200 HPLC system (Agilent, USA) interfaced with a 3200 Q TRAP® mass spectrometer (Applied Biosystems, Foster City, Canada) equipped with a TurboIonSpray® source. Cliquid software (Applied Biosystems, Foster City, Canada) was used to control equipment, to coordinate data acquisition, and to analyse data.

MPH, RA and the internal standard d_6-amphetamine were separated under gradient elution using a RESTEK Allure PFP Propyl 5 μm 50 mm × 2.1 mm internal diameter analytical column (Restek, USA) with a RESTEK Allure PFP Propyl 10 mm × 2.1 mm guard cartridge. The column oven was set at 40 °C. The mobile phase consisted of solvent A (0.02% formate in water) and solvent B (0.02% formate in acetonitrile). The flow rate was set at 0.5 mL/min. The initial condition was 90% solvent A and 10% solvent B. A linear gradient was performed with mobile phase B increasing from 10% to 90% within 10 min and held for 1 min. The mobile phase was returned to the initial condition and re-equilibrated for 2 min. The total analysis time was 13.5 min.

The mass spectrometer was operated in the positive ion mode with curtain gas, Gas 1 and Gas 2 flow rates of 20, 40 and 70 psi, respectively. The ion spray voltage was 4000 V and the source temperature was 500 °C. Data acquisition was performed via selected reaction monitoring (SRM). The ions representing the $[M+H]^+$ species for MPH, RA and the internal standard d_6-amphetamine were selected in the first mass spectrometer (mass analyser, MS1) and dissociated with nitrogen gas to form specific product ions, which were subsequently monitored by the second mass spectrometer (mass analyser, MS2). The optimised precursor-to-product ion transitions monitored for MPH were *m*/*z* 234.1 (Q1) and 84.1 and 56.1 (Q3). For RA, the optimised precursor-to-product ion transitions monitored were *m*/*z* 220.1 (Q1) and 84.1 and 56.2 (Q3). For d_6-amphetamine the optimised precursor-to-product ion transitions monitored were *m*/*z* 142.2 (Q1) and 125.1 and 93.1 (Q3) (Fig. 2).

2.3. Standards

A standard stock solution of MPH was prepared by dissolving 1 mL of 1 mg/mL commercial standard into 50 mL methanol to give a final concentration of 20 mg/L.

A standard stock solution of RA was prepared by dissolving 2 mg of commercial standard in 100 mL methanol to give a final concentration of 20 mg/L.

The stock internal standard was prepared by dissolving 1 mL (1 mg/mL) d_6-amphetamine diluted to 50 mL with methanol. This was further diluted (1:50) in acetonitrile.

The urine calibration curves for MPH and RA were constructed using internal standard, by spiking drug-free human urine with standard solutions at concentrations of 5, 10, 25, 50, 100, 500, 1000, 5000 μg/L, giving a calibration range of 5–5000 μg/L for both MPH and RA.

2.4. Sample preparation

100 μL of the internal standard, d_6-amphetamine was added to 100 μL of each of blank, standard, quality control or patient urine samples. To this mixture, 800 μL of distilled, deionised water was added to give 1 mL final volume and injected onto the column.

2.5. Validation

The standard curves were the plot of the peak area ratios (analyte/internal standard) of MPH and RA versus the corresponding concentrations of MPH and RA. The linearity of the standard curves was evaluated using $1/x$-weighted linear regression analysis. To evaluate the assay recoveries and matrix effects, three sets of standards were prepared using a modification of the method of Matuszewski et al. [12] for MPH and RA.

Absolute recoveries at each concentration were measured by comparing the peak area of MPH or RA and the internal standard in urine standards to those in the blank urine samples which were spiked post dilution at the corresponding concentrations ($n = 6$) [absolute recovery = (peak area of analyte from the spiked urine sample)/(peak area of analyte from the post dilution spiked blank urine sample) × 100%]. The matrix effects were assessed by comparing the peak area of MPH or RA and the internal standard from the post dilution spiked blank urine samples with the peak area of MPH or RA and the internal standard from the standard solution at the same concentration in the mobile phase ($n = 6$).

Quality control samples were prepared by spiking drug free human urine with both MPH and RA at concentrations of 100, 500 and 1000 μg/L.

Quality control was assessed by analysis of six samples at each concentration on the same day (intra-day) and of one sample at each concentration on six different days (inter-day). Bias was determined as the measured minus the actual concentration, expressed as a percentage of the actual concentration. Imprecision was measured as intra- and inter-day coefficients of variation. The limit of quantification for this assay was defined as the lowest concentration of MPH or RA that could be detected with acceptable accuracy and precision ($n = 6$) (according to the US Food and Drug Administration guidance for bioanalytical method validation, the mean value determined at the lowest concentration should not deviate by more than 20% of the actual value, and the precision determined at the lowest concentration should not exceed 20% of the coefficients of variation [13]).

The effects of freezing and thawing on the concentrations of MPH and RA were studied using QC samples at 100, 500 and 1000 μg/L, which were subjected to four freeze–thaw cycles before analysis. The stability of urine QC samples at −20 °C was evaluated by concentration analysis at weekly intervals for 6 months. The stability of the stock standard solutions of MPH and RA at −20 °C for 6 months was evaluated by comparing the response with that of the freshly prepared standard solutions. The stability of the processed samples at 4 °C (the temperature of the autosampler) for 3 days was evaluated by comparing the results with the original results. In all cases, MPH and RA were considered to be stable as long as degradation was <10% of the concentration at day 0.

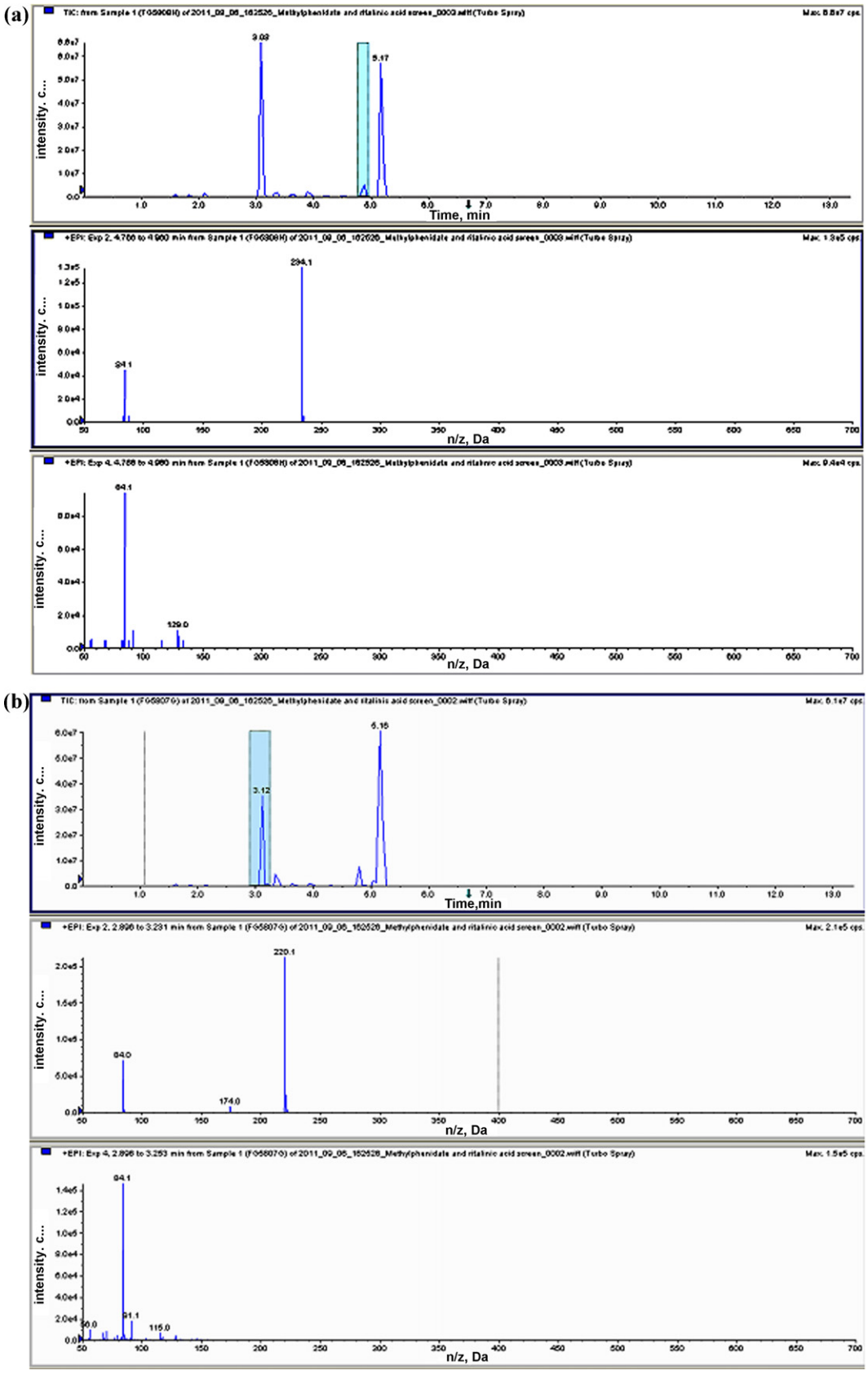

Fig. 2. Product ion mass spectra of (a) $[M+H]^+$ for MPH, (b) $[M+H]^+$ for RA and (c) $[M+H]^*$ for d_6-amphetamine. Top pane shows chromatogram of total ion count (TIC); middle pane shows spectrum at collision energy (CE) 20 V; bottom pane shows spectrum at CE 50 V.

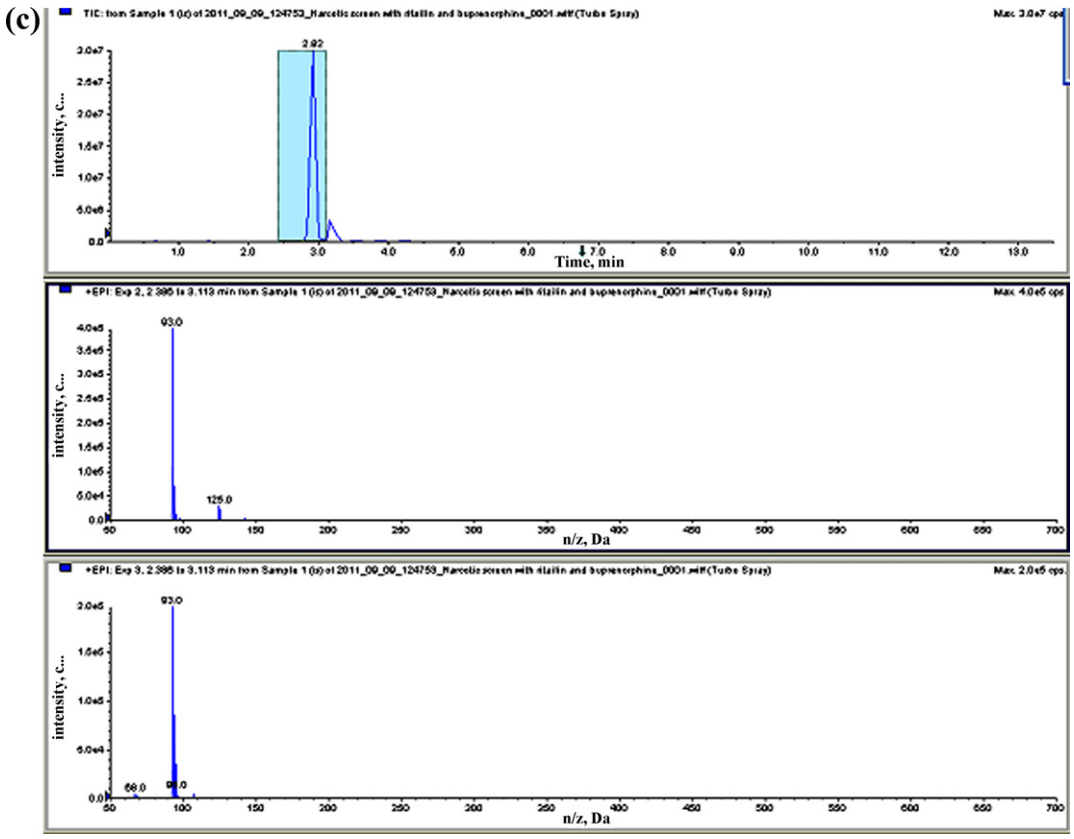

Fig. 2. (*Continued*).

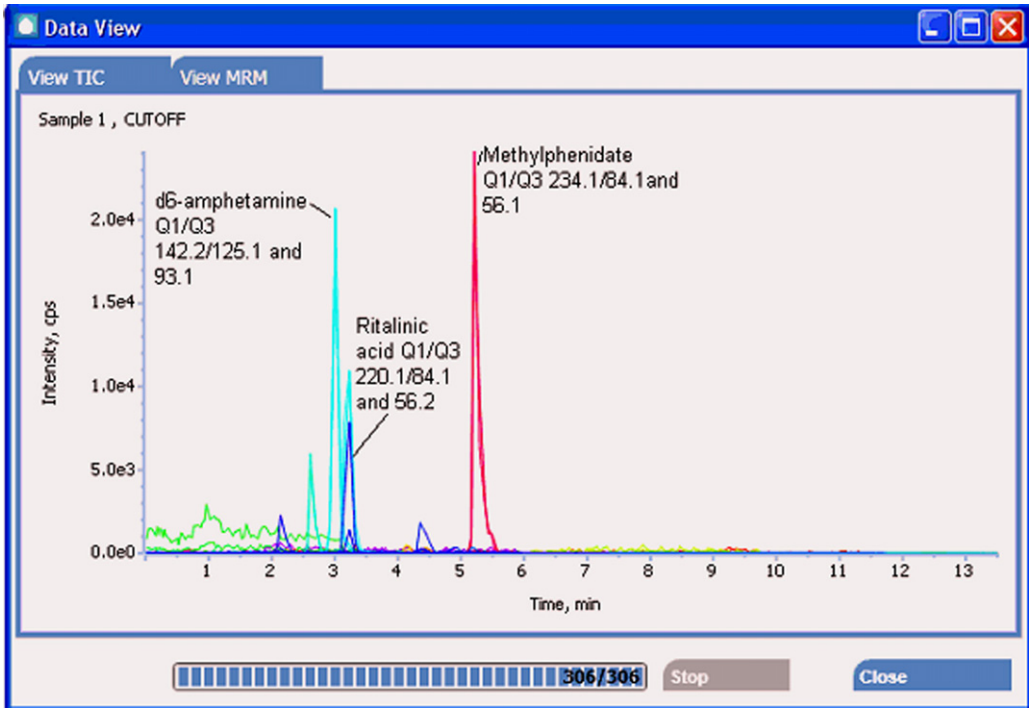

Fig. 3. Representation of SRM monitored Q1/Q3 transitions for a urine sample from a patient on MPH.

3. Results and discussion

3.1. Mass spectrometry and chromatography

The MS/MS parameters were optimised to produce maximum responses for MPH, RA and the internal standard, d_6-amphetamine, using electrospray ionisation in the positive ion mode. The protonated molecular ions $[M+H]^+$ were m/z 234.1, 220.1 and 142.2 for MPH, RA and internal standard, respectively.

The product ion spectra of $[M+H]^+$ for three compounds are shown in Fig. 2. The transitions yielding the most abundant product ions were 234.1 → 84.1 and 234.1 → 56.1 for MPH, 220.1 → 84.1 and 220.1 → 56.2 for RA and 142.2 → 125.1 and 142.2 → 93.1 for d_6-amphetamine (Fig. 3).

MPH, RA, and the internal standard were separated from matrix components using a RESTEK Allure PFP Propyl column and a mobile phase consisting of 0.02% formate and acetonitrile. Gradient elution was chosen to obtain better resolution and to enhance separation efficiency. Under the chromatographic conditions employed, the retention times were 3.01, 3.82 and 5.79 min for the internal standard, RA and MPH, respectively (Fig. 4).

Drug free human urine samples from more than six different sources of the same matrix were tested for interference. MPH, RA and the internal standard peaks were free of interference from any other peaks present in the urine blanks (Fig. 4).

No carry-over was observed by injecting an extract of blank urine immediately following the three injections of the highest calibration standard.

Our laboratory has analysed over 10,000 urine samples from our client catchment, including samples from acute hospital admissions, drug rehabilitation clinics, mental health organisations and workplace employment screening. We have not seen any interference from other drugs of abuse, prescription medications or varying urine matrices.

3.2. Sample preparation

The higher sensitivity of the LC–MS/MS technique, compared with the ELISA methodology previously used in our laboratory, allowed us to use a very small volume of urine (100 μL) for the quantification of MPH and RA in urine.

Dilution with water is the simplest and most rapid method of urine sample preparation for the measurement of drug concentrations by LC–MS/MS. MPH, RA and the internal standard were free of interference from endogenous compounds in the urine. To ensure long term performance, the guard column cartridge was changed every 200–300 injections. The analytical column demonstrated no deterioration of performance after more than 2000 injections.

3.3. Method validation

Urine standard curves of MPH and RA, prepared with d_6-amphetamine as the internal standard were linear ($r > 0.997$) over the concentration range of 5–5000 μg/L. The intercept with the y-axis was not significantly different from zero. The typical standard curves were as follows: $y = 0.0029x - 0.1492$ ($r = 0.9976$) for MPH and $y = 0.0039x + 0.1448$ ($r = 0.9976$) for RA.

The lower limit of quantification (LLOQ) for both MPH and RA was 100 μg/L in urine, at which the mean values were within ±20% of the spiked values and the intra- and inter-day coefficients of variation were <7.6% for MPH and <7.8% for RA (Tables 1 and 2).

The limit of detection for both MPH and RA was 5 μg/L. This was the lowest detectable concentration with a signal to noise ratio >3:1.

There was no constant direction to the bias (i.e. + or −) for urine QC samples and the mean values were within ±10% of the spiked values. Imprecision was acceptable, as indicated by both intra- and inter-day coefficients of variation of <7.8% at all concentrations from MPH and RA (Tables 1 and 2).

Table 1
Intra-day assay variance of the determination of MPH and RA in urine ($n = 6$).

Sample	Concentration spiked (μg/L)	Concentration found (μg/L) (mean ± SD)	Bias (%)	Imprecision CV (%)
MPH				
QC1 (LOQ)	100	95.4 ± 1.9	−4.6	2.0
QC 2	500	511.1 ± 26.4	2.2	5.2
QC 3	1000	1028.39 ± 46.1	2.8	4.5
RA				
QC1 (LOQ)	100	106.8 ± 4.76	6.8	4.4
QC 2	500	522.8 ± 13.3	4.6	2.5
QC 3	1000	1031.3 ± 54.5	3.1	5.3

The absolute recoveries of MPH and RA at concentrations of 100, 500 and 1000 μg/L were similar and consistent, with the mean values >90%. The absolute recovery of the internal standard d_6-amphetamine at the concentration employed was 82%.

The matrix effects were assessed by comparing the response of MPH, RA and the internal standard from the spiked post dilution blank urine extracts with the response of standard solution at the same concentration in the mobile phase [matrix effect = (peak area of analyte spiked in post dilution blank urine)/(peak area of analyte spiked in mobile phase) × 100%]. A value of 100% indicates that the responses in the mobile phase and in spiked post dilution urine were the same and no absolute matrix effect is observed. A value of >100% indicates an ionisation enhancement and a value of <100% indicates an ionisation suppression. The matrix effects (mean ± SD%) determined at concentrations 100, 500 and 1000 μg/L for MPH were 130 ± 13.0, 90 ± 16.9 and 127 ± 11.9%, respectively, and for RA were 125 ± 8.9, 95 ± 10.3 and 120 ± 18.0%, respectively. The matrix effect for the internal standard d_6-amphetamine was 140 ± 20%. The results showed that there was ionisation enhancement but this was accounted for by the internal standard.

MPH and RA were found to be stable in urine for at least four freeze–thaw cycles when stored at −20 °C. The urine QC samples at concentrations of 100, 500 and 1000 μg/L were stable for at least 6 months at −20 °C. The stock standard solutions MPH and RA were shown to remain stable for at least 6 months at −20 °C. The processed samples were stable for at least 3 days at 4 °C.

3.4. Application of the assays

The method presented is currently being used in our laboratory service to measure the concentrations of MPH and RA in urine for monitoring compliance of patients on MPH therapy and

Table 2
Inter-day assay variance of the determination of MPH and RA in urine ($n = 6$).

Sample	Concentration spiked (μg/L)	Concentration found (μg/L) (mean ± SD)	Bias (%)	Imprecision CV (%)
MPH				
QC1 (LOQ)	100	103.2 ± 7.7	3.2	7.5
QC 2	500	525.3 ± 12.6	5.1	2.4
QC 3	1000	1086.7 ± 58.2	8.7	5.4
RA				
QC1 (LOQ)	100	103.3 ± 7.6	3.3	7.4
QC 2	500	525.9 ± 16.6	5.2	3.2
QC 3	1000	1029.8 ± 79.4	3.0	7.7

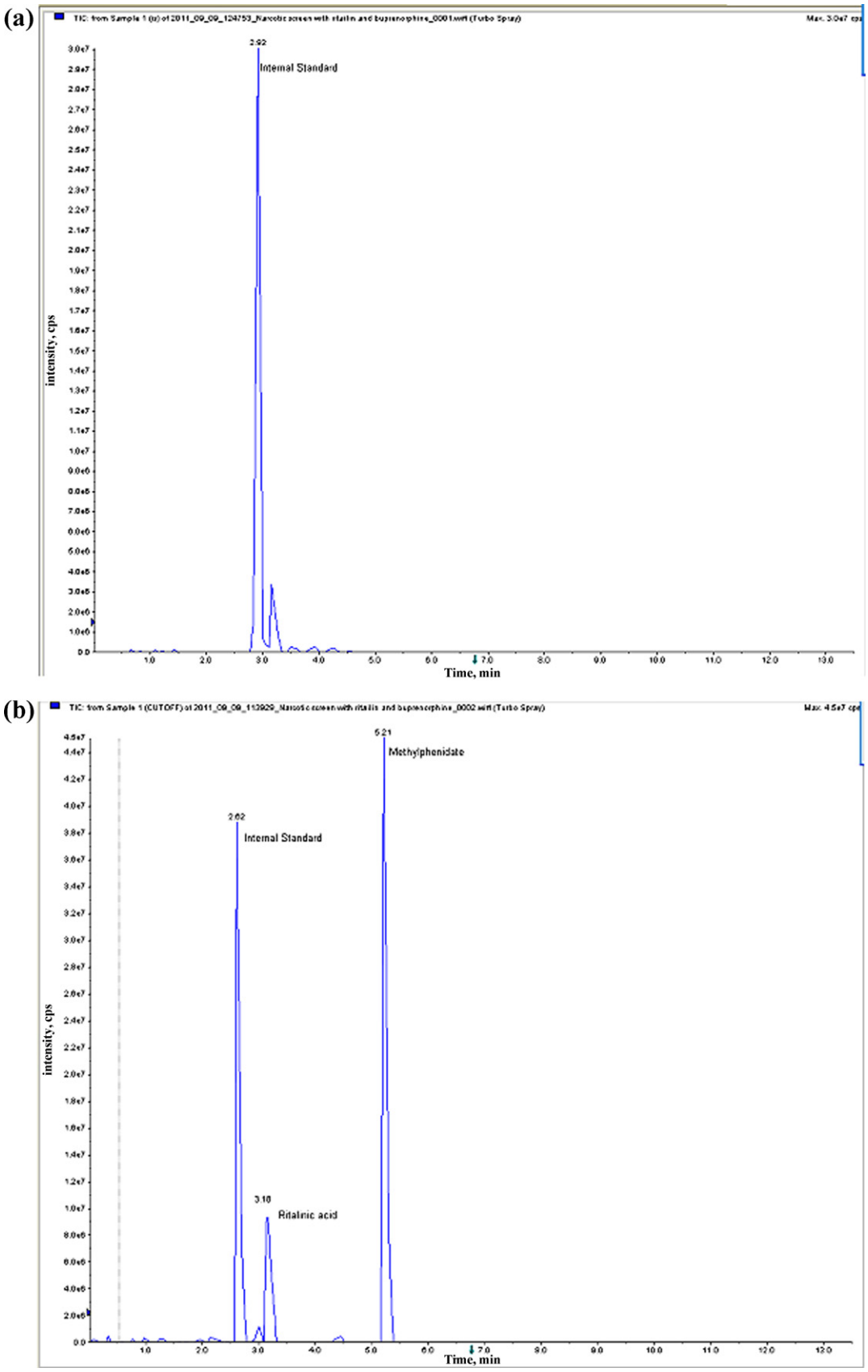

Fig. 4. Representative chromatograms of (a) blank urine with d_6-amphetamine, (b) urine sample spiked with MPH and RA at 100 μg/L and (c) urine sample from a patient on MPH.

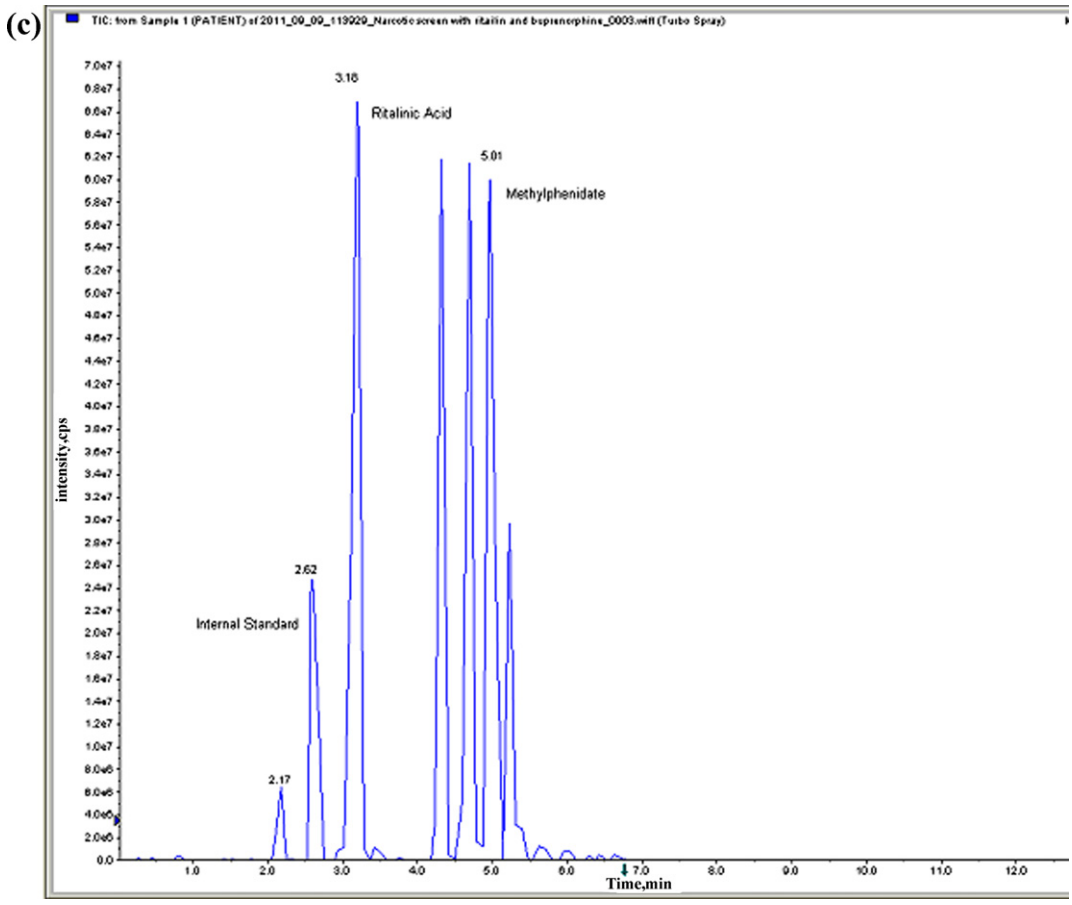

Fig. 4. (*Continued*)

also as a drug of abuse screening method. To ensure the accuracy and reproducibility of the method, we have participated in AUSTOX, a quality assurance programme administered by the Toxicology Unit at Pacific Laboratory Medical Services, Sydney and the Royal Collage of Pathologists of Austrailasia quality assurance programme, RCPA-QAP. The monthly returned reports have shown the performance has always been acceptable with 100% compliance.

4. Conclusions

A validated LC–MS/MS method for the determination of MPH and RA has been described. The method has proven to be rapid, sensitive, specific, accurate and precise, and is currently being used in routine clinical service to monitor compliance of patients on MPH therapy and to also screen for abuse.

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