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# High-throughput selected reaction monitoring liquid chromatography–mass spectrometry determination of methylphenidate and its major metabolite, ritalinic acid, in rat plasma employing monolithic columns

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**Abstract**

This work presents a high-throughput selected reaction monitoring (SRM) LC–MS method for the determination of methylphenidate (MPH), a central nervous stimulant, and its de-esterified metabolite, ritalinic acid (RA) in rat plasma samples. A separation of these two compounds was achieved in 15 s by employing a 3.5-ml/min flow-rate, a porous monolithic column and a TurboIonSpray source compatible with relatively high flow-rates. In addition, a relatively fast autosampler and a new data acquisition system resulted in a time lag of less than 17 s between consecutive injections. Overall, 768 protein-precipitated rat plasma samples (eight 96-well plates) containing both MPH and RA were analyzed within 3 h and 45 min. The partial method validation described in this report included an assessment of linearity, intra and inter-assay precision and accuracy, and method robustness. Deuterated internal standards for the target compounds, $d_3$-MPH and $d_5$-RA, were employed. The calibration curves ranged from 0.1 to 50 ng/ml for MPH and from 0.5 to 50 ng/ml for RA. The limit of quantification (LOQ) for MPH and RA was 0.1 and 0.5 ng/ml, respectively. For both analytes, the intra- and inter-assay precision (relative standard deviation, % C.V.) and accuracy (relative error) did not exceed 15% for the quality control samples (QCs) QC1, QC2 or QC3 (0.3, 1.5 and 40 ng/ml for MPH and 0.15, 15 and 40 ng/ml for RA) for either analyte and did not exceed 20% at the lower limit of quantitation (LOQ) level. No carry-over from the autosampler was detected. The retention times remained constant throughout the experiment. Baseline resolution of MPH and RA was consistently observed throughout the plates analyzed. The described method demonstrates the feasibility for employing monolithic HPLC columns to effect rapid bioanalytical SRM LC–MS analysis of representative biological samples.
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*Keywords:* Methylphenidate; Ritalinic acid

## 1. Introduction

The ever increasing need for speed and efficient use of time in the pharmaceutical and other fields places a demand for the development of faster,

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higher throughput analytical procedures. Liquid chromatography coupled with tandem mass spectrometry (LC–MS–MS), due to its sensitivity and selectivity, facilitates the development of high-throughput methods of analysis. LC–MS–MS has revolutionized the strategies and success of modern drug discovery and development in the pharmaceutical industry. However, efforts continue to further improve the sample throughput capabilities of modern techniques and strategies.

The development of combinatorial chemistry techniques and other modern strategies for discovering new drug entities has led to an increasing number of potential drug candidates and has created a need for rapid bioanalyses for toxicological and pharmacokinetic studies. The rapid, trace level quantitative determination of drugs and their metabolites remains a challenge which is often driven by the need for same-day turnaround of results from large numbers of biological samples. Conventional techniques such as LC–UV or LC–fluorescence (LC–FL) because of their limited selectivity or sensitivity, often require slower HPLC gradients and relatively long analysis times for separation of the compounds of interest from interfering matrix components.

Because of its high sensitivity and selectivity, selected reaction monitoring (SRM) LC–MS has become the method of choice for the quantitative determination of analytes in biological samples [1–4]. The unique selectivity of SRM LC–MS has allowed for simpler sample preparation procedures and shorter analysis times, which aid in the development of high-throughput methods of analysis.

A relatively fast SRM LC–MS technique with a separation time of less than 1 min was first demonstrated in 1986 [5]. In 1999, we described a fast SRM LC–MS method for the determination of benzodiazepines in human urine (1000 samples/12 h) [6]. The quantitative determination of selective estrogen receptor modulators (SERMs) in less than 30 s (2000 samples/day) was described in the following year [7]. Both cases required the use of four autosamplers coupled to one chromatographic column, rendering these methods relatively equipment-intensive and complicated. Recently, idoxifene (another SERM) and its major pyrrolidinone metabolite [8] were measured using a single autosampler configured for sequential injection of eight samples in rapid sequence followed by a single rinsing step [8]. Although the separation was obtained in less than 10 s, a total sample-to-sample cycle time of 23 s was required to accommodate autosampler rinsing and downloading of the mass spectrometer acquisition parameters. Throughput can also be increased via methods which require little or no sample pretreatment. These ‘‘direct injection’’ methods generally employ on-line clean-up [9–11].

MPH (α-phenyl-2-piperidineacetic acid methyl ester) is a central nervous system stimulant which is mainly prescribed for children and adolescents with attention deficit disorder (ADD) and in the treatment of narcolepsy [11]. It acts by inhibition of the presynaptic uptake of dopamine and norepinephrine and by promoting the synaptic release of dopamine [12]. MPH is rapidly metabolized in both humans and animals, predominantly giving a hydrolyzed product commonly known as ritalinic acid (RA, α-phenyl-2-piperidineacetic acid) [13,14]. The chemical structures for MPH and RA are shown in Fig. 1 along with the deuterated internal standards employed in this work.

This report presents a high-throughput bioanalytical method for the quantitative determination of MPH and its major metabolite, RA, in rat plasma. We have previously reported the achiral determi-

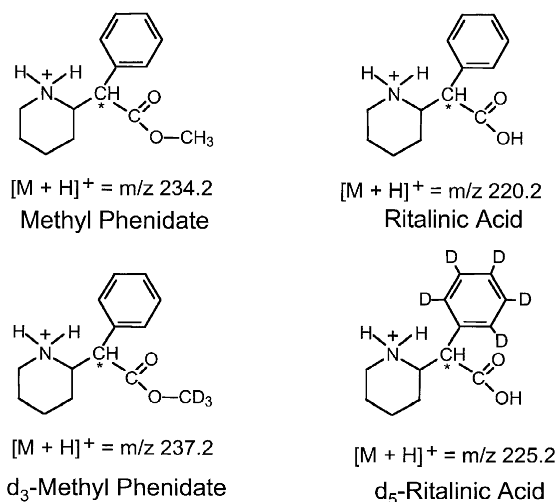


Fig. 1. Structures of MPH and RA and their corresponding deuterated internal standards. Also shown are the corresponding $m/z$ values for the respective protonated molecules.

nation of this compound in human urine via capillary electrophoresis–mass spectrometry using an ion trap mass spectrometer [15]. Although it is sometimes important to employ a bioanalytical method capable of determining both enantiomers of methyl phenidate [16] it is often sufficient to measure the combined substances as one analyte. This latter strategy was employed in this work.

The use of a single autosampler [8] capable of injecting a new sample within 15 s, a novel commercially available monolithic HPLC column, a high mobile phase flow-rate [17,18], and an efficient data handling system allow completion of the SRM LC–MS separation and analysis of each sample in less than 15 s permitting sample-to-sample cycle time of 17 s. Accordingly, 768 samples contained in eight 96-well plates were analyzed in 3 h and 45 min. This level of performance may be of use in those instances where large numbers of samples in a single batch require rapid turn-around from the bioanalytical laboratory.

## 2. Experimental

### *2.1. Materials and methods*

HPLC-grade acetonitrile was obtained from J.T. Baker (Phillipsburg, PA). Formic acid (88%, double distilled) was from GFS Chemicals (Columbus, OH). Deionized water was generated in-house with a Barnstead Nanopure II filtration system (Boston, MA). Standards of MPH and RA were from USP (Rockville, MD) while $d_3$-methylphenidate ($d_3$-MPH) and $d_5$-ritalinic acid ($d_5$-RA) were from Isotec (Miamisburg, OH). The $d_0$ contribution to both deuterated internal standards was less than 0.1% as determined by abbreviated full-scan acquisition over the protonated molecule region of each compound. Acid citrate dextrose rat plasma was obtained from Lampire Biological Laboratories (Coopersburg, PA). Protein precipitation was carried out in 1.2-ml polypropylene 96-well plates purchased from Phenix Research Products (Hayward, CA) and sealed with Cap Mats from Matrix Technologies (Hudson, NH). Sample evaporation and reconstitution was performed in 0.5-ml polypropylene 96-well plates from VWR (South Plainfield, NJ) which were sealed with capmats of the same brand. The Chromolith Performance RP-18e 4.6 mm I.D.×100 mm, Chromolith SpeedROD RP-18e 4.6 mm I.D.×50 mm, and Chromolith Flash RP-18e 4.6 mm I.D.×25 mm columns were provided by Merck (Darmstadt, Germany).

### *2.2. Preparation of standards, QCs and I.S. solution*

Separate stock solutions containing 1 μg/ml of MPH or RA in acetonitrile were obtained from Advion BioSciences (Ithaca, NY). A stock standard plasma solution containing 50 ng/ml of both analytes was generated by spiking control rat plasma with the MPH and RA stock solutions. The total volume of acetonitrile added to the plasma was judged to be insufficient to affect its properties. This plasma solution was used as the highest concentration standard, and was diluted serially with control rat plasma to obtain additional standards at concentrations ranging from 0.1 to 50 ng/ml for MPH and 0.5 to 50 ng/ml for RA. Standards were prepared at ten concentration levels for MPH and eight concentration levels for RA, and arranged from lowest to highest concentration at the beginning and the end of each plate. Each set was preceded and followed by two blank samples (no analytes) and one double blank sample (no analytes or internal standards). It must be noted that all standard samples were prepared in an ice water bath to inhibit the enzymatic hydrolysis or chemical conversion of MPH to RA. The standard samples were maintained in ice until the enzymatic reaction was quenched by the addition of 20 μl of 1% formic acid (see Sample preparation section). As reported by Ramos et al. [16] the R,R′ isomer of MPH hydrolyzes rapidly, while the S,S′ isomer is reported to be stable for more than 24 h at room temperature. Although this method does not attempt to distinguish between these two analytes, precautions were taken to minimize these changes during sample handling.

QC samples were prepared similarly at four concentration levels with six replicates for each level placed randomly on each 96-well plate. The QC concentrations corresponded to the following regions of the standard calibration range: lower limit of quantification (LOQ), lower quartile (QC1), mid-

range (QC2), and upper quartile (QC3). The respective QC concentrations were 0.3, 1.5 and 40 ng/ml for MPH and 0.15, 15 and 40 ng/ml for RA. Each LOQ QC was preceded by a blank sample. Because incurred samples were not available, ''mock'' samples were prepared at various concentrations using the stock standard plasma solution. In order to mimic unknown samples, these mock samples were randomly placed among the QC samples, and were not labelled with any indication of their analyte concentrations. The deuterated analogues of MPH and RA were used as internal standards (I.S.). Their structures are shown in Fig. 1. An I.S. solution containing 0.5 ng/ml $d_3$-MPH and 4 ng/ml $d_5$-RA in $H_2O$–acetonitrile (9:1) was generated from 2-μg/ml stock solutions. Thus, each 96-well plate consisted of 20 standards, 24 QCs (six replicates each at four concentration levels), and 38 unknowns as well as 14 blanks or double blanks, placed before and after each set of standards and before each LOQ sample. For the analysis of actual clinical or experimental samples it is likely that approximately two-thirds of the plate would consist of unknown samples, with the remaining wells containing standards, QC samples and blanks.

### *2.3. Method validation*

Method validation included an investigation of method linearity, limit of quantification, intra- and inter-assay precision and accuracy, and robustness of the method. The results from individual plates were used to determine the intra-assay precision and accuracy while the inter-assay precision and accuracy were determined using the results from all eight plates. We sought a method which would meet accuracy and precision criteria similar to those recommended by the United States Food and Drug Administration [19]. The intra- and inter-assay accuracy of the calibration standards should be within 15% of the theoretical concentration (20% at the limit of quantification, LOQ). The inter-assay precision (relative standard deviation, % C.V.) of the standards should be less than 15% (20% at LOQ). The coefficient of determination ($r^2$) should be >0.98 for each calibration curve. Similarly, the intra- and inter-assay accuracy of each QC sample should be within 15% of the expected concentration, and the corresponding intra- and inter-assay precision should be less than 15%. These experiments constitute a partial method validation since recovery and freeze–thaw stability were not determined. The use of deuterated analogue internal standards minimizes the impact of any variations in recovery which may exist.

### *2.4. Sample preparation*

Rat plasma samples (25 μl) were pipetted manually into 1.2-ml polypropylene 96-well plates using a 200-μl Pipetteman (Gilson, Middleton, WI). Prior to sample acidification, an ice bath was used to maintain all sample tubes and 96-well plates at ~0 °C. Pipetting of solvents and transfer of the sample supernatant was performed robotically using a Tomtec Quadra 96 model 320 robotic pipettor (Hamden, CT). All samples were first treated with 20 μl of 1% formic acid solution in $H_2O$–acetonitrile (9:1) to inhibit enzymatic conversion or chemical reaction of MPH to RA. The plate was then mixed on a vortex mixer for 1 min. The internal standard solution (20 μl) was added to all samples except the double blanks to which 20 μl of a $H_2O$–acetonitrile (9:1) solution was added. After agitating the plate on a vortex mixer for 1 min, protein precipitation was performed by adding 400 μl of acetonitrile to all samples. The plate was centrifuged at 2100 *g* for 10 min at room temperature using an Eppendorf 5810R centrifuge with a four-plate rotor (Brinkmann Instrument, Westbury, NY). The supernatant from each well was then robotically transferred to a fresh 0.5-ml polypropylene 96-well plate via the Tomtec pipettor and evaporated to dryness in a 40 °C water bath under a gentle stream of nitrogen using an in-house-constructed blow-down apparatus. The dried samples were then reconstituted with 100 μl water; the plate was then agitated on a vortex mixer for 1 min and centrifuged for 5 min at 2100 *g* (room temperature). The samples were transferred to the LC–MS system for high-throughput analysis by SRM LC–MS employing a Chromolith Flash RP-18e 4.6 mm I.D.×25 mm HPLC column.

### *2.5. Liquid chromatography and mass spectrometry*

The SRM LC–MS analyses were performed using a Shimadzu LC-2010 (Shimadzu, Columbia, MD) integrated HPLC system including a degasser, high

speed autosampler, column oven and low pressure pump, coupled to an AB MDS Sciex (Concord, Ont., Canada) API 4000 triple quadrupole mass spectrometer. The chromatographic separation was performed isocratically on a 4.6×25 mm $C_{18}$ column (Cromolith; Merck, Darmstadt, Germany). The mobile phase consisted of 25% acetonitrile and 75% water containing 0.1% formic acid. The injection volume was 25 μl. The flow-rate was maintained at 3.5 ml/min and a post-column split was established to deliver 2.1 ml/min to the mass spectrometer with the remaining (1.4 ml/min) directed to waste. The column was maintained at 35 °C and the mobile phase was preheated using an integrated column preheater.

SRM LC–MS analyses were performed using the TurboIonSpray Source (AB MDS Sciex, Concord, Ont., Canada) operated in the positive ion mode and maintained at 650 °C. The mass spectrometer tuning parameters were optimized for MPH and RA by infusing a solution containing 0.5 mg/ml of both analytes at a flow-rate of 10 μl/min into the mobile phase (2.1 ml/min) using a post-column ''T'' connection. The nebulizer and TurboIonSpray gases were set at 70 and 80 p.s.i., respectively. The optimized TurboIonSpray voltage was set at 2000 V and the CID gas was 6 (arbitrary units). The collision energy was 29 eV. The following transitions were monitored in the SRM mode: MPH, $m/z$ 234.2>84.1; $d_3$-MPH, $m/z$ 237.2>84.1; RA, $m/z$ 220.2>84.1; $d_5$-RA, $m/z$ 225.2>84.1. The dwell time was set to 75 ms for each of the four transitions. The mass spectrometer was operated at unit mass resolution (peak width at half-height set at 0.7 Da) for both Q1 and Q3. Data were acquired with the Sciex Analyst software, version 1.1 (AB MDS Sciex, Concord, Ont., Canada).

## 3. Results and discussion

Full-scan (Q1) and collision-induced dissociation (CID) mass spectra for MPH (A) and RA (B) are shown in Fig. 2. These data show the protonated molecules for methylphenidate and its metabolite, RA, at $m/z$ 234.2 and $m/z$ 220.2, respectively (Fig. 2A and B) as well as their relatively simple CID fragmentation behavior (Fig. 2C and D, respectively). The $m/z$ 84.1 ion is the predominant product ion from each of the protonated molecules and thus provides the majority of the ion current. Maximum SRM LC–MS sensitivity is afforded using these transitions.

Fig. 3A–C shows the SRM LC–MS chromatograms corresponding to the separation of MPH and RA (analytical standards, 0.062 ng each injected on-column) using 100-, 50- and 25-mm column lengths, respectively. All other conditions remained the same in these experiments. A separation of the target drug (MPH) and its metabolite (RA) was obtained within 15 s using a high flow-rate (3.5 ml/min) and a short 25-mm column length (Fig. 3C). This was possible due to the combination of high mobile phase flow-rate and minimum back pressure afforded by the monolithic column, together with the data acquisition and management capabilities of the AB MDS Sciex API 4000 triple quadrupole mass spectrometer. The monolithic columns employed are based on sol–gel chemistry which incorporates a macroporous (~2-μm pore diameter) channel structure giving rise to a low back pressure and therefore permitting a high flow-rate. The mesoporous (13 nm) substructure affords high chromatographic efficiency [17,18,20,21]. This HPLC column displayed robust performance in that over 2000 biological extracts were injected onto this column before it was replaced. Chromatographic peak shape, symmetry, and HPLC separation efficiency were maintained as documented by comparison with data provided with the product insert received with the new column. Since this project employed only one monolithic column generously provided by Merck, we cannot comment on the batch-to-batch performance of these new monolithic HPLC columns. In addition, the AB MDS Sciex API 4000 triple mass spectrometer was equipped with a TurboIonSpray source (Turbo V™ source) which supports high HPLC flow-rates with increased sensitivity and minimized chemical background noise. The ''Analyst'' software also minimizes the acquisition delay between consecutive data files, allowing rapidly sequenced injections.

Sample preparation involved a simple protein precipitation procedure with acetonitrile and was performed directly in 96-well plates. All plasma samples were acidified with 20 μl of 1% formic acid in order to inhibit the hydrolysis of MPH to RA. Prior to this addition, all samples were maintained in

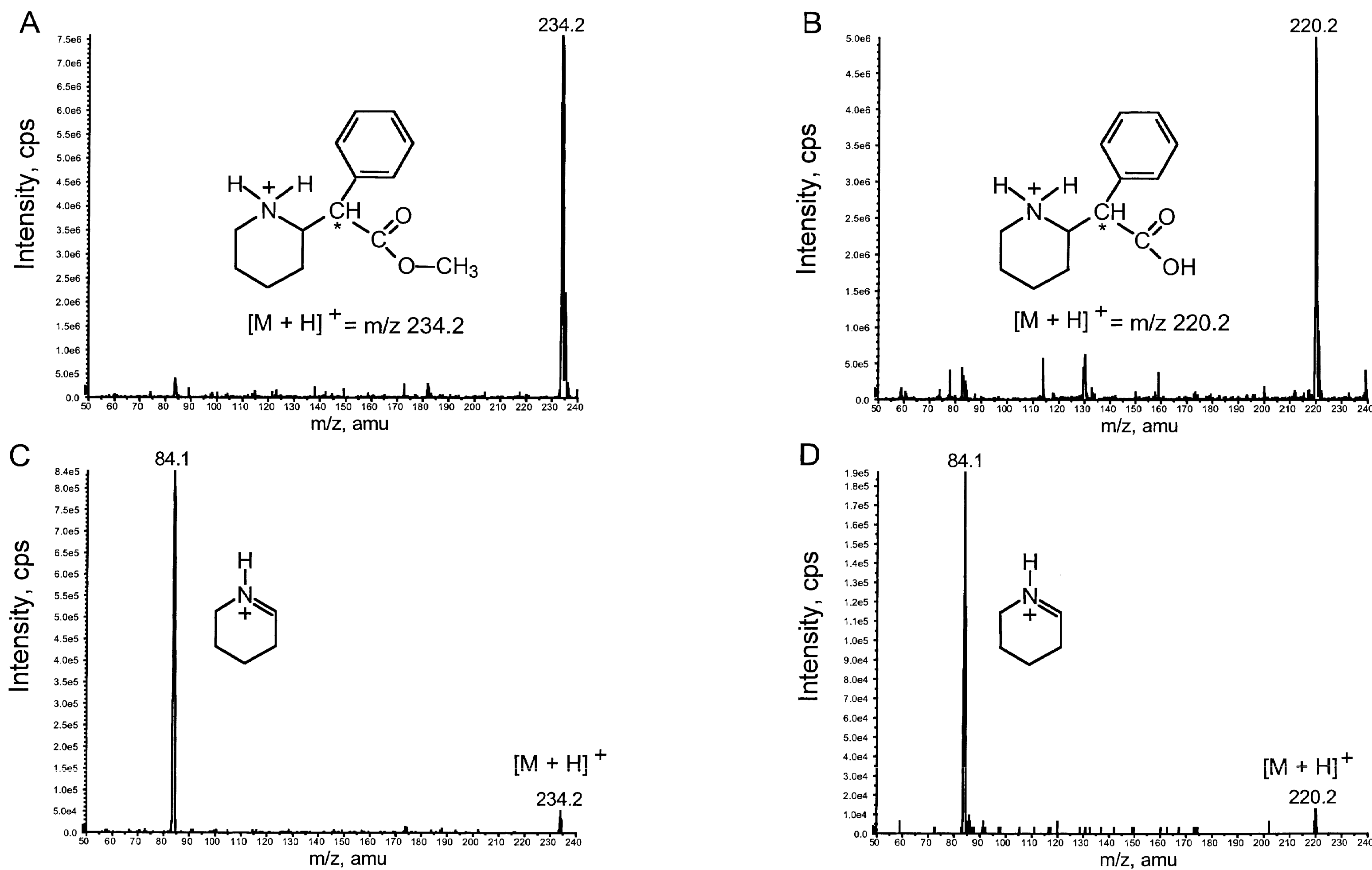


Fig. 2. Full-scan (A and B) and CID (C and D) mass spectra for MPH and RA, respectively. The *m/z* for the protonated molecules are shown in each figure, along with the corresponding masses for the major product ion at *m/z* 84.1 in C and D. $d_3$-MPH displayed a protonated molecule at *m/z* 237.2 with a product ion at *m/z* 84 while the $d_5$-RA displayed a protonated molecule at *m/z* 225.2 with a product ion at *m/z* 84.

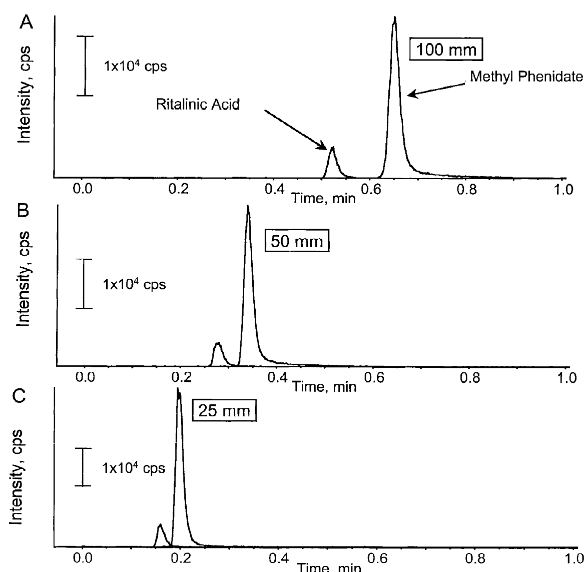


Fig. 3. SRM LC–MS chromatograms showing analysis times for the rapid separation of MPH and RA analytical standards using monolithic columns of lengths: (A) Chromolith Performance RP-18e 4.6 mm I.D.×100 mm; (B) Chromolith SpeedROD RP-18e 4.6 mm I.D.×50 mm; (C) Chromolith Flash RP-18e 4.6 mm I.D.×25 mm. The HPLC flow-rate in each experiment was 3.5 ml/min, 25/75 acetonitrile/0.1% formic acid, with a post-column split of 2.1 ml/min to the LC–MS interface. In each experiment 25 μl of a solution containing 2.5 ng/ml of each compound (0.062 ng each) was injected.

an ice bath in order to slow this reaction. Using this procedure, the analysis of supernatant from protein-precipitated plasma fortified with MPH revealed no presence of RA (data not shown). The simplicity and the rapidity of the protein precipitation process makes it well suited for high-throughput analysis. The protein-precipitated plasma supernatant samples were sufficiently clean to be analyzed directly by SRM LC–MS. Indeed, no increase in column pressure and no clogging of connecting tubing were detected after the analysis of hundreds of these plasma samples.

Nonetheless, this relatively simple sample pretreatment process does little to remove endogenous substances which may coelute or otherwise interfere with the SRM LC–MS detection of the targeted analytes. It is well known that reduced electrospray ion current can result from "ion suppression" due to the presence of endogenous components or even the metabolites of the parent drug if insufficient chromatography is employed [22–24]. This reduction in ion current in the presence of coeluting compounds is reportedly due to a change in colligative properties in the droplet solution in contrast to gas-phase charge stripping processes [25]. In any event, very short run times such as those reported in this work can suffer from these effects. Inspection of the ion current profiles shown in Fig. 4 shows a lowering of the baseline in the ~0.11–0.15-min retention time region. It was important to establish a chromatographic separation with sufficient retention of the targeted analytes such that they did not elute in this retention time region [24]. Post-column infusion experiments employing a mixture of MPH and RA were performed following the previously reported strategy [24,25]. These experiments were performed to determine where in the chromatographic profile the

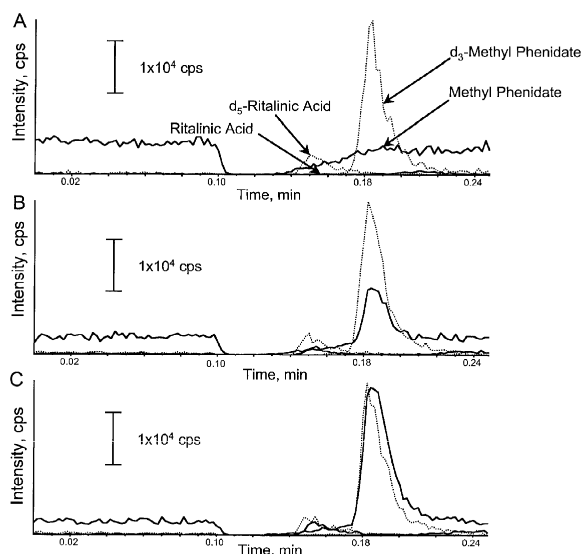


Fig. 4. SRM LC–MS chromatograms showing representative data for the analysis of the blank, LOQ, and low QC samples from the described work. (A) Blank plasma sample containing deuterated internal standards for MPH and RA in the absence of the non-labeled target analytes. (B) LOQ plasma sample containing the respective deuterated internal standards for MPH and RA in addition to the two non-labeled target analytes fortified at the LOQ which was 0.1 ng/ml for MPH and 0.5 ng/ml for RA. (C) Low QC plasma sample containing the respective deuterated internal standards for MPH and RA in addition to the two non-labeled target analytes fortified at the LOQ which was 0.3 ng/ml for MPH and 1.5 ng/ml for RA.

target analytes would suffer the least from co-elution of the biological sample matrix components when a representative sample of control matrix extract was injected into the chromatographic system while monitoring the respective transitions for the target analytes (data not shown). It is also important to note the potential for later eluting components in the endogenous mixture which can sometimes cause ion current signal suppression of target analytes in subsequent injections. Therefore, a systematic evaluation of these potential factors should be assessed early in a method development protocol. This is especially important when very short run times are employed as reported herein which can compromise the ability of the chromatographic process to adequately separate matrix and other interferences from the target analytes. The method described in this report took these issues into account.

There are also related issues which must be addressed when rapid analyses such as those described herein are employed for the analysis of post-dose biological samples. In particular, if conjugates of RA are formed in vivo and are not chromatographically separated from the parent acid, they may fragment in the pre-mass analysis region of the API mass spectrometer to form the same protonated molecule of the analyte. This could potentially interfere with the quantification of the parent acid, for example. These rather polar metabolites may be difficult to chromatographically separate under the described very short analysis times employed in this work, so one must be careful to avoid this situation. In this work the nozzle-skimmer region of the API 4000 mass spectrometer was tuned to a low voltage difference to minimize this possibility. However, since only fortified biological samples were analyzed in this work, it cannot be said with confidence that these conditions would preclude this possibility. To ensure the very short analysis time employed in this work does not contribute to fragmenting such polar conjugated metabolites selectivity in post-dose samples should be demonstrated. Alternatively one could consider a cross-validation using post-dose samples of the assay with an established assay to ensure that similar results are obtained in both cases. This was not done in this work.

High-throughput analysis required the use of an autosampler which enabled the injection of a different sample every 15 s. To reach this goal, samples were injected without an autosampler needle rinse step. To characterize the level of carry-over, a blank sample routinely followed the highest concentration standard (50 ng/ml). A typical chromatogram is shown in Fig. 4A which displays no significant interference from the non-deuterated target analytes. The ion current profiles shown in Fig. 4B show the LOQ plasma sample containing the respective deuterated internal standards for MPH and RA in addition to the two non-labeled target analytes fortified at the LOQ which was 0.1 ng/ml for MPH and 0.5 ng/ml for RA at an approximate retention time of 0.15 min. It is important to note that although the chromatographic peaks for RA and its co-eluting deuterium labeled internal standard appear as weak chromatographic peaks in this figure, integration of the peak areas for these peaks was straightforward. When these peaks were subjected to individual normalization (data not shown) the automated peak area integration of the API 4000 data system easily produced the peak areas for these seemingly weak ion current profiles. Panel C in Fig. 4 shows the SRM LC–MS ion current profile for the low QC plasma sample containing the respective deuterated internal standards for MPH and RA in addition to the two non-labeled target analytes fortified at the LOQ which was 0.3 ng/ml for MPH and 1.5 ng/ml for RA. As indicated above in spite of the apparent weak intensity for the peaks for RA area integration for the peaks at an approximate retention time of 0.15 min was straightforward.

The overall experiment consisted of analyzing 768 plasma extracts contained in eight 96-well plates. Each plate contained two replicates of the calibration curve. The first set of standards was placed at the beginning of each plate in order of increasing concentration, while the second set of standards was placed in the same sequence at the end of the plate. Also, six replicates of each QC sample, e.g. four concentration levels, as well as blanks, double blanks and unknown samples, were distributed randomly throughout the plate as described in the Experimental section.

Fig. 5A–C shows SRM LC–MS chromatograms of three equivalent QC samples (low QC, 0.3/ml of MPH and 1.5 ng/ml of RA). In Fig. 5A is shown the 8-min sample (sample 31, plate 1), Fig. 5B the 92-min sample (sample 368, plate 4) and Fig. 5C the 188-min sample (sample 752, plate 8). These chro-

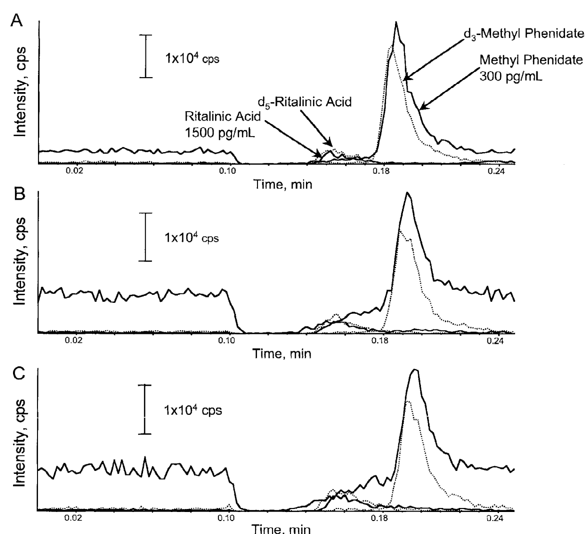


Fig. 5. SRM LC–MS chromatograms showing representative data for the analysis of low QC samples from the described work taken at three different times over the course of the rapid sequential analysis of 768 samples (eight 96-well plates). Each of these samples contained the respective deuterated internal standards for MPH and RA in addition to MPH at 0.3 ng/ml and RA at 1.5 ng/ml. (A) Low QC sample 31 analyzed at 7.75 min into the analysis period. (B) Low QC sample 368 analyzed at 92 min into the analysis period. (C). Low QC sample 752 analyzed at 188 min into the analysis period.

matograms show that the chromatographic retention times, HPLC peak separation, HPLC peak shape as well as the peak heights, remained constant throughout the nearly 4-h analysis time. However, it must be noted that the apparent chemical noise level, especially for MPH, increased with the number of injections. Although the increased chemical noise level did not affect the quality of the quantitative results (see below), the LOQ could have been affected if more samples had been analyzed. Thus if significantly more than eight 96-well plates were to be analyzed with this method, it might be helpful to employ a gradient elution of the monolithic HPLC column after the first eight plates or even perhaps after each plate to remove elevated levels of potentially interfering chemical constituents which may accumulate in the chromatographic system.

### *3.1. Method linearity*

The calibration range for the described method ranged from 0.1 to 50 ng/ml of rat plasma for MPH and 0.5 to 50 ng/ml for RA. A standard calibration curve was constructed for each plate using a weighted ($1/x*x$) linear regression based upon the concentration versus peak area ratio (analyte/internal standard). The deuterated analogues ($d_3$-MPH and $d_5$-RA) were used as internal standards for the respective analytes. A representative calibration curve is shown in Fig. 6 for both MPH (Fig. 6A) and RA (Fig. 6B). The lower limit of quantification (LOQ) was defined as the lowest point of the calibration curve for which the acceptance criteria were met, and corresponded to 0.1 ng/ml of MPH and 0.5 ng/ml of RA. Fig. 4B and C presents representative chromatograms for the analysis of an LOQ and a QC1 sample, respectively.

### *3.2. Precision and accuracy*

The intra-assay precision and accuracy were determined for each plate at three QC levels. All values met the acceptance criteria at QC1, QC2, and QC3 (vide supra). As an example, the results obtained for the last plate (plate 8) all met the normal acceptance criteria for a bioanalytical method [19] (Table 1). It was observed that the accuracy obtained for QC1 nearly exceeded the limits of the acceptance criteria (114.2% for MPH and 85.2% for RA). This could be explained by the higher level of background detected for later injections. If a large batch of samples (four or more 96-well plates) were analyzed, a programmed gradient elution of the column might be required, for example, between each plate. This could preclude excessive accumulation of retained matrix components which might interfere with the chromatographic performance of the column. The inter-assay precision and accuracy were obtained using the values of all eight plates and were within the acceptance criteria for QC1, QC2 and QC3. The results are presented in Table 2. Overall, the analysis of 768 samples took 3 h and 45 min.

## 4. Conclusions

A partially validated high-throughput SRM LC–MS bioanalytical method was developed for the determination of MPH and its major RA metabolite in rat plasma using a short monolithic HPLC column. The combination of new technologies (short

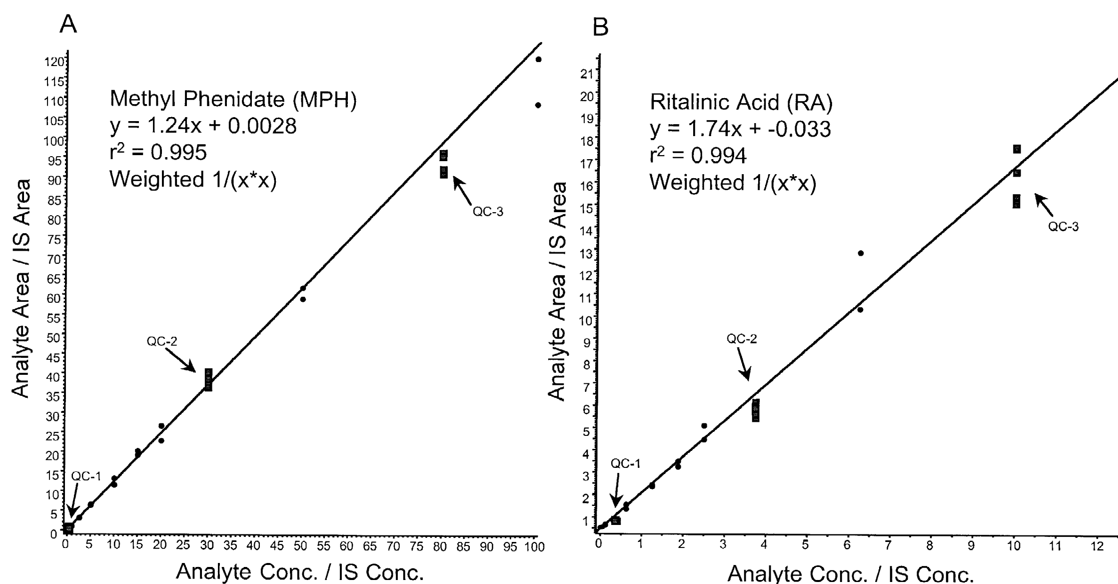


Fig. 6. Representative calibration curves for (A) MPH and (B) RA.

Table 1
Intra-assay precision and accuracy for plate 8 ($n=6$)

| Expected QC conc. (pg/ml) | C.V. (%) | Accuracy (%) |
|---|---|---|
| *Methylphenidate* | | |
| 300 (QC1) | 6.1 | 114 |
| 15 000 (QC2) | 4.1 | 103 |
| 40 000 (QC3) | 2.7 | 93 |
| *Ritalinic acid* | | |
| 1500 (QC1) | 12.4 | 85 |
| 15 000 (QC2) | 4.7 | 95 |
| 40 000 (QC3) | 6.9 | 99 |

Table 2
Inter-assay precision and accuracy for all plates ($n=48$)

| Expected QC conc. (pg/ml) | C.V. (%) | Accuracy (%) |
|---|---|---|
| *Methylphenidate* | | |
| 300 (QC1) | 8.7 | 111 |
| 15 000 (QC2) | 6.3 | 102 |
| 40 000 (QC3) | 6.2 | 91 |
| *Ritalinic acid* | | |
| 1500 (QC1) | 10.7 | 94 |
| 15 000 (QC2) | 9.5 | 101 |
| 40 000 (QC3) | 6.8 | 100 |

cycle injection, monolithic column, column heater, highly sensitive mass spectrometer and modern acquisition software) allows the limits of high-throughput bioanalytical analysis to be extended. This capability is helpful if one wants to provide a very short turnaround time (e.g. the same afternoon) for a small batch of samples, or much faster than usual turnaround time for a large batch of samples.

Sample preparation by protein precipitation is adequate for high-throughput analyses, and produced plasma extracts which were sufficiently clean to avoid clogging of tubing, increased system back pressure, or gradual deterioration of chromatographic results. It is worth noting that cleaner sample extracts could be obtained by employing solid-phase extraction (SPE) or liquid–liquid extraction (LLE) [26] sample preparation techniques. Either of these techniques would likely improve the robustness of high-throughput SRM LC–MS analyses and minimize matrix suppression or related problems. However, sample preparation ‘‘bottlenecks’’ still remain. For example, the time required for manually pipetting 768 plasma samples into the eight 96 well-plates is not compatible with high-throughput analysis and needs to be addressed by collecting the original biological samples directly into 96-well plates, or by

employing appropriate robotics to first aliquot biological samples from vials or tubes into the same.

A total of eight 96-well plates (768 samples), including standards, QC samples, unknowns, blanks and double blanks, were analyzed in 3 h and 45 min. A single analyst performed all these experiments which included aliquoting the biological samples into the plates, sample preparation and SRM LC–MS analysis. In a preferred situation a sample preparation team would provide the prepared samples to the LC–MS laboratory for rapid analysis and return of the results. Despite a gradual increase in chemical background noise observed in the SRM LC–MS ion current profiles, the method was shown to be sufficiently robust to produce quality results that meet current method validation acceptance criteria.

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