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Direct plasma liquid chromatographic–tandem mass spectrometric analysis of granisetron and its 7-hydroxy metabolite utilizing internal surface reversed-phase guard columns and automated column switching devices

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Abstract

An alternative on-line automated sample enrichment technique useful for the direct determination of various drugs and their metabolites in plasma is described for rapid development of highly sensitive and selective liquid chromatographic methods using mass spectrometric detection. The method involves direct injection of plasma onto an internal surface reversed-phase (ISRP) guard column, washing the proteins from the column to waste with aqueous acetonitrile, and backflushing the analytes onto a reversed-phase octyl silica column using switching valves. The analytes were detected using a tandem mass spectrometer operated in selected reaction monitoring (SRM) mode using atmospheric pressure chemical ionization (APCI). Use of two ISRP guard columns in parallel configuration allowed alternate injections of plasma samples on these columns for sample enrichment and shortened the column equilibration and LC–MS–MS analysis times, thereby increasing the sample throughput. The total run time, including both sample enrichment and chromatography, was about 6 min. Using this technique, an analytical method was developed for the quantitation of granisetron and its active 7-hydroxy metabolite in dog plasma. Granisetron is a selective 5-HT3 receptor antagonist used in the prevention and treatment of cytostatic induced nausea and vomiting. Recovery of the analytes was quantitative and the method displayed excellent linearity over the concentration ranges tested. Results from a three day validation study for both compounds demonstrated excellent precision (1.3–8.7%) and accuracy (93–105%) across the calibration range of 0.1 to 50 ng/ml using an 80 μl plasma sample. The automated method described here was simple, reliable and economical. This on-line approach using ISRP columns and column switching with LC–MS–MS is applicable for the quantification of other pharmaceuticals in pharmacokinetic studies in animals and humans which require high sensitivity.

Keywords: Granisetron; 7-Hydroxygranisetron

1. Introduction

Quantitative analysis of therapeutic compounds and their metabolites in pre-clinical and clinical biological samples by various analytical techniques is a vital function in drug discovery and development. Typically these analytical methods involve extraction of drugs and their metabolites from a biological matrix (such as plasma, serum or urine), separation most often by high-pressure liquid chro-

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matography (HPLC) and detection using one of several readily available detectors, such as ultraviolet absorbance, fluorescence, electrochemical or mass spectrometric detectors. Although advances have been made in automation of HPLC systems, sample preparation procedures which greatly impact on method development, sample analysis time and overall sample throughput, are frequently performed manually using traditional isolation methods.

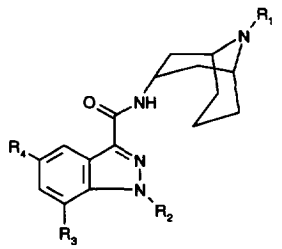
The effect of sample preparation times on method development and sample analysis have become more pronounced since the introduction of LC–MS–MS for routine quantitative analysis. This technique has greatly increased the number of samples that can be analyzed in a day by reducing the analysis cycle time (frequently <5 min) and decreased the time required for method development through its inherently high sensitivity and specificity. In order to use LC–MS–MS optimally, large numbers of samples are prepared for analysis using one of several traditional sample preparation techniques which are amenable to LC–MS–MS analyses including solid-phase extraction (SPE), liquid–liquid extraction, and protein precipitation. Precipitation of proteins from biological samples prior to LC–MS–MS analysis is a quick procedure, but, because the samples become diluted in the process and are still relatively dirty, it is not amenable to methods requiring very high sensitivity. Liquid–liquid extraction is also typically faster than SPE approaches and is useful for high sensitivity assays, but is not readily automated and is not applicable to more polar analytes. Of all of these sample preparation methods, SPE approaches, which readily concentrate analytes, offer the broadest range of applicability in terms of fulfilling high sensitivity requirements on a wide range of chemical structures. Robotic equipment for automating SPE is commercially available, but is expensive, slow in processing samples and generally requires human intervention at various stages of sample preparation. In order to eliminate the disadvantages associated with off-line sample preparation methods, we have considered various on-line sample preparation techniques which require little or no sample manipulation, and are compatible with LC–MS–MS systems.

A form of SPE which is available commercially, but has not received general application, is an on-line process involving Restricted Access Media (RAM) columns [1–5]. Internal Surface Reversed Phase (ISRP) is one type of Restricted Access Media, which is compatible with the direct injection of plasma samples without prior sample preparation. Proteins in plasma samples are excluded by the outer hydrophilic phase from entering the inner hydrophobic phase of the ISRP columns and pass through the columns without clogging [6–11] while the small lipophilic analytes are retained by the inner reversed phase surface. Column switching devices have also been successfully used for transferring the analytes from RAM columns to reversed phase analytical columns for trace analysis of various analytes [12,13]. This combination of short RAM guard columns for plasma protein removal and column switching devices for analyte transfer offered a simple on-line sample preparation technique that could be easily interfaced with the LC–MS–MS system to develop high sensitivity assays. This on-line process has resulted in excellent clean-up and enrichment of the analytes of interest and is particularly well suited for ultra-high sensitivity LC–MS–MS applications. In order to demonstrate the utility of this approach, a highly sensitive and specific analytical method utilizing an internal standard was developed for the simultaneous quantitation of granisetron and its active 7-hydroxy metabolite (7OH-G) in dog plasma. Granisetron (BRL 43694, Figure 1) {endo-1-methyl-N-(9-methyl-9-azabicyclo [3.3.1]non-3-yl)-1H-indazole-3-carboxamide} is a selective 5-HT3 receptor antagonist which has been developed for the prevention and treatment of nausea and emesis induced by cytostatic therapy [14–17].

2. Materials and methods

2.1. Chemicals

Granisetron hydrochloride (G, Fig. 1), 7-hydroxy-granisetron hydrochloride (7OH-G, Fig. 1), metabolites A (BRL 46540, Fig. 1), B (BRL 43110, Fig. 1) and internal standard (I.S., BRL 43704, Fig. 4) were supplied by SmithKline Beecham Pharmaceuticals (Worthing, West Sussex, UK). Glacial acetic acid, ammonium acetate and HPLC-grade acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA).



Compound	R_1	R_2	R_3	R_4
Granisetron	N-CH_3	CH_3	H	H
7OH-G	N-CH_3	CH_3	OH	H
Metabolite A	N-H	CH_3	H	H
Metabolite B	N-CH_3	H	H	H

Fig. 1. Structures of granisetron and its unconjugated metabolites.

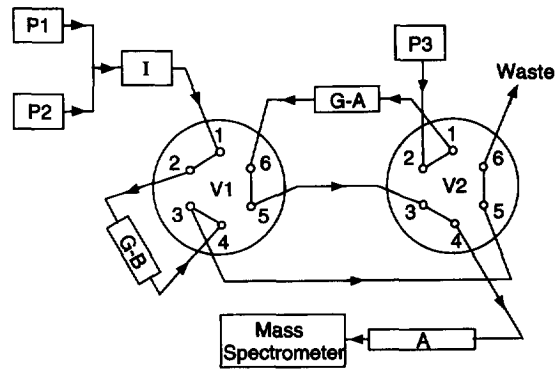


Fig. 2. Schematics of the on-line sample preparation system. Both valves V1 and V2 are in **on** position. P1 and P2=gradient pump for loading solvent, I=injector, V1 and V2=switching valves, G-A and G-B=ISRP guard columns, P3=mobile phase pump, A=analytical column.

2.2. Standard solutions

The stock standard solutions of granisetron, 7OH-G, and I.S. were prepared by dissolving appropriate amounts of the compounds in water to give a final base concentration of 1 mg/ml. The solutions were stable for 4 months when stored at 4°C. Working aqueous standard solutions of granisetron and 7OH-G at concentrations of 100, 10 and 1.0 μg/ml were prepared daily by appropriate dilutions of stock solutions with water. The working internal standard solution was prepared by appropriate dilution of the stock solution of I.S. with water to give a solution concentration of 100 ng/ml. This solution was stable for 1 month when stored at 4°C.

2.3. Sample preparation

Dog plasma samples (100 μl) were transferred to HPLC autosampler vials and centrifuged at 2000 *g*.

2.4. High-performance liquid chromatography

The HPLC system (Fig. 2 and Fig. 3) consisted of a column-switching device (WAVS, Waters Associates, Milford, MA, USA), a high-pressure gradient semi-micro solvent delivery system (Model LC-6200A intelligent pumps, Hitachi Instruments, Danbury, CT, USA), an isocratic HPLC pump (Model 116, Beckman Instruments, Palo Alto, CA, USA) an autosampler with an autoaddition feature (Model 715 UltraWisp, Waters Associates) and a Sciex API III triple quadrupole mass spectrometer (Perkin Elmer/Sciex Corporation, Thornhill, Ontario, Canada). Chromatographic separations were carried out on a 5 cm×4.6 mm I.D. 5 μm octyl silica

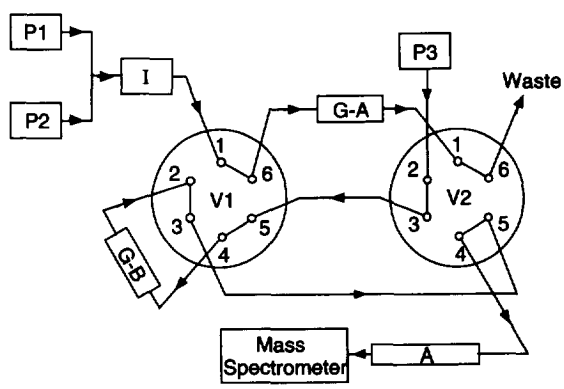


Fig. 3. Schematics of the on-line sample preparation system. Both valves V1 and V2 are in **off** position. P1 and P2=gradient pump for loading solvent, I=injector, V1 and V2=switching valves, G-A and G-B=ISRP guard columns, P3=mobile phase pump, A=analytical column.

column (Inertsil, Metachem, CA, USA) which was preceded by 1.5 cm×1 mm I.D. octyl silica guard column (Opti-guard, Optimize Technologies, Portland, OR, USA).

2.5. Column-switching configuration

The heart of the column switching device consisted of two pneumatically controlled six port Rheodyne valves (V1 and V2), which could be individually switched between **on** (Fig. 2) and **off** (Fig. 3) positions. Two 10 mm×3.0 mm I.D. Internal Surface Reversed Phase (ISRP GFF II, G-A and G-B) guard columns (Regis Chemical, Morton Grove, IL) and one 5 cm×4.6 mm I.D. 5-μm octyl silica column (A, Inertsil, MetaChem Technologies, Torrance, CA, USA) were connected through the two valves of the column switching device as shown in Fig. 2 and Fig. 3. The high-pressure solvent gradient system (pumps P1 and P2) was connected to switching valve V1 through the injector (I) and was used to load plasma samples onto ISRP guard columns A or B as well as to wash and re-equilibrate the ISRP columns. The initial loading solvent, composed of water–acetonitrile (95:5, v/v), was pumped at a rate of 1.0 ml/min. An additional isocratic pump (P3) was connected to valve V2 of the switching device. Pump P3 supplied the chromatographic mobile phase which backflushed the analytes from the guard columns on to the analytical column (A) and eluted the analytes into the mass spectrometer. The chromatographic mobile phase was 0.05 *M* ammonium acetate pH 5.0–acetonitrile (73:27, v/v) which was pumped at a rate of 1.0 ml/min.

2.6. Automated sample enrichment and chromatography using a column switching device

The most elegant feature of the present system, in addition to the direct injection of plasma samples without pre-treatment, was the alternating pre-column enrichment technique which dramatically reduced the analysis cycle time. Initially, both valves V1 and V2 were set to the **on** position (Fig. 2) such that guard column G-B was vented to waste. With the valves set to this position, guard column G-B was conditioned with loading solvent (water–acetonitrile, 95:5, v/v) from the gradient solvent system (pump P1 and P2) in preparation for the autosampler to inject a plasma sample. Also during this stage, the mobile phase (0.05 *M* ammonium acetate pH 5.0–acetonitrile, 73:27, v/v) from pump P3 flowed into the mass spectrometer through guard column G-A and analytical column A. In a typical chromatographic run, the autosampler sequentially drew 10 μl of internal standard solution (I.S., 100 ng/ml) and 80 μl of (centrifuged) plasma through the use of its auto addition feature, and injected this mixture onto the ISRP guard column G-B while activating the gradient and timed events of the gradient pump system. The timed events from the gradient HPLC pumps (P1 and P2) controlled the column switching device and, thereby, the flow path of the entire on-line sample preparation system. Valves V1 and V2 remained at this position (**on**) for 2 min, during which the plasma proteins were vented to waste from the G-B column. At the end of the 2 min period, both valves V1 and V2 were switched to the **off** position (Fig. 3), allowing the elution mobile phase from Pump P3 to backflush the analytes from the G-B guard column. The analytes were separated on the analytical column and detected by the mass spectrometer. Also during this **off** stage, the G-A guard column was washed and re-equilibrated in preparation for the next sample. The concentration of acetonitrile from gradient pumps P1 and P2 was increased to 20% in 0.1 min in order to wash the G-A guard column and was then cycled back to the loading solvent conditions (water–acetonitrile, 95:5, v/v) after 3 min. The G-A guard column was then ready to receive the next sample. The valves remained in this position while the next sample was injected onto the G-A guard column and the cycle was repeated according to the schematics shown in Fig. 3. The autoinjector injected plasma samples at 6 min intervals alternatively on guard columns G-B and G-A and the actual chromatographic run time (the time that the guard column was switched in line with the analytical column and mass spectrometer) was 4 min.

2.7. Mass spectrometric parameters

The Sciex API III triple quadrupole mass spectrometer was operated in selected reaction moni-

toring (SRM) mode using positive atmospheric pressure chemical ionization (APCI) with a discharge needle current (DI) of 3 μA. The nebulizing gas (air) pressure was 75 p.s.i. ($5.2 \cdot 10^5$ Pa) and the auxiliary and curtain gas flows were 1.0 and 1.1 l/min, respectively. The orifice potential was set at 60 V. The heated nebulizer temperature was set at 450°C and the sample pump was operated with a source delta value of 0.6 in. of H_20. Ions were collisionally activated at an energy of 35 eV and the collision gas (argon) thickness was $250 \cdot 10^{13}$ molecules cm^{-2}. Product ion mass spectra obtained by infusing a standard solution of granisetron, 7OH-G and the internal standard (BRL 43704, I.S.) are shown in Fig. 4. In order to quantify granisetron and 7OH-G, the

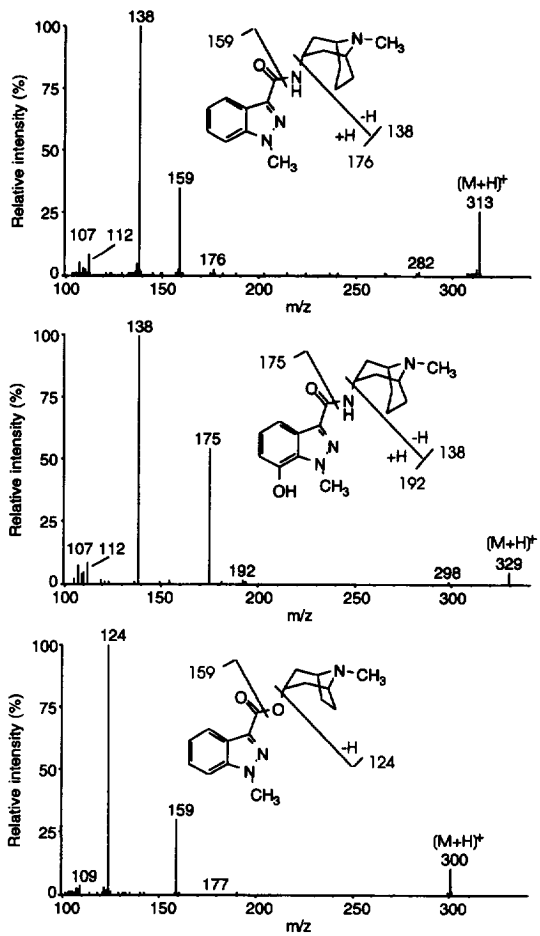


Fig. 4. Product ion mass spectra for granisetron (top), 7OH-G (middle), and the internal standard (bottom) obtained using atmospheric pressure chemical ionization.

mass spectrometer was set to select the $[M+H]^+$ at m/z 313 for granisetron, m/z 329 for 7OH-G and m/z 300 for I.S. via the first quadrupole mass filter (Q1). Signals for product ions at m/z 138, 138 and 124 for granisetron, 7OH-G and I.S., respectively, were monitored via the third quadrupole mass filter (Q3). The dwell time for each ion was 200 ms and the mass filters were tuned to unit resolution. Ions were detected with an electron multiplier operating in pulse-counting mode by counting every pulse (count by one). SRM chromatographic data were collected using Sciex RAD software. Calibration curves and sample concentrations were determined using Sciex MacQuan software.

2.8. Validation procedures

Four pools of plasma precision samples containing 0.1, 0.2, 5, and 50 ng/ml each of granisetron and 7OH-G were prepared by adding appropriate volumes of standard solutions to drug-free dog plasma. These plasma samples were stored at −20°C until the analysis was performed. Seven replicate samples from each pool were analyzed on three separate days. Concentrations of analytes in plasma were determined by comparison with calibration lines constructed from samples containing 50, 25, 10, 5, 2, 1, 0.5, 0.2, 0.1 and 0.05 ng/ml each of granisetron and 7OH-G prepared on the day of analysis by serially diluting a plasma sample containing 100 ng/ml each of granisetron and 7OH-G. A weighted ($1/y$) linear regression was used to construct a calibration line for the peak area ratio of analyte to internal standard versus analyte concentration. From the data obtained, inter-day precision (determined as coefficients of variation of daily means), intra-day precision (determined as mean of the daily coefficients of variation) and mean accuracy were calculated.

3. Results and discussion

Although many successful methods have been reported using RAM columns and conventional HPLC detectors [18–34], specificity of these detection systems has frequently limited the general utility of this approach for high sensitivity assays.

On the other hand, the high specificity provided by the mass spectrometric detection appears to be particularly suited for such on-line methods as demonstrated by the highly sensitive LC–MS–MS assay that is described here for quantitation of granisetron and its 7-hydroxy metabolite (7OH-G) in dog plasma. Using this on-line sample preparation system, the plasma sample was first injected onto the ISRP guard column. The plasma proteins and other polar substances, which were not retained by the ISRP column, were directed to waste using a switching device while less polar small molecules (including analytes) were retained by the column. After removal of endogenous proteins, the analytes of interest were back-flushed from the ISRP column onto the analytical column through the switching valve for further separation and eluted into the mass spectrometer for quantitation. Use of two ISRP guard columns in parallel configuration allowed alternate injections of plasma samples on these columns for sample enrichment, minimized the build up of plasma proteins on the guard column, and shortened the column equilibration and LC–MS–MS analysis times, thereby increasing the sample throughput.

3.1. Isolation of analytes from plasma using ISRP columns

Although different types of RAM columns are commercially available, including semi-permeable surface (SPS) and internal surface reverse phase (ISRP) columns, the ISRP columns provided quantitative, reproducible recovery of the analytes of interest from plasma. The performance of the two ISRP guards were identical as demonstrated by the C.V. values of peak areas of various analytes and the internal standard that were obtained from the injection of three replicate plasma samples on these two columns (Table 1). Several parameters were found to be important for the optimal performance of the ISRP columns. The concentration of acetonitrile in the loading solvent was critical for the retention of granisetron and its metabolites on the ISRP columns. Concentrations of acetonitrile ranging from 3–5% yielded quantitative recoveries of granisetron and 7OH-G, presumably by disrupting the interaction of the analytes with plasma proteins. Increasing the acetonitrile concentration above 5% (v/v) substantially reduced the recovery of both the analytes and internal standard. If the loading solvent concentration of acetonitrile exceeded 25% (v/v), protein precipitation in the ISRP column was probable and eventually could effect the overall performance of the analytical column and the mass spectrometer. The pH of the loading solvent was also critical. Optimum performance of the ISRP guard columns was observed when the loading solvent pH was between 6 to 7.5 to maintain the proper charge state on the ISRP particles to exclude proteins [6]. However, the pH of the elution solvent did not effect the stability or longevity of the ISRP column as long as the pH remained between 2.5–7.5. Using 5% aqueous acetonitrile solution as the loading solvent (during injection of plasma samples), the performance of the ISRP guard columns was unchanged even after injection of 150 plasma samples (on two guard columns).

Table 1
Reproducibility of ISRP columns G-A and G-B

Column	Peak area		
	7OH-G 5 ng/ml	I.S. 12.5 ng/ml	Granisetron 5 ng/ml
G-A	38412	157404	127059
G-B	39441	155379	124851
G-A	41657	153272	125105
G-B	40927	155293	122641
G-A	36784	148844	120939
G-B	37557	147943	117910
Mean	39130	153023	123084
S.D.	1908	3827	3304
C.V. (%)	4.88	2.50	2.68

Six plasma samples that contained 5 ng/ml of each granisetron and 7OH-G and IS (12.5 ng/ml) were alternatively injected on G-A and G-B columns.

3.2. Recovery

Attempts were made to calculate the overall recovery of analytes and the internal standard from the ISRP guard columns by injecting separately aqueous standard solutions and spiked plasma standards, which contained equivalent concentrations of analytes, onto the ISRP guard columns according to the methods described in the experimental section. The peak areas of the analytes were lower for the aqueous standards than for the plasma standards,

yielding artificially high recoveries for all the analytes (>100%). These high recoveries appeared to be the result of poorer retention or poorer recovery of the analytes by the ISRP guard columns from the aqueous solutions than from plasma samples. Blank plasma samples indicated that interference from common ions was not a problem; however, the possibility of enhanced ionization of the analyte induced by an interference can not be ruled out unequivocally.

3.3. Sensitivity, linearity and selectivity

Fig. 5 displays SRM chromatograms obtained simultaneously from a single injection of an aqueous sample containing, granisetron, its unconjugated metabolites (7OH-G, metabolite A, metabolite B) and the internal standard (I.S.). The chromatographic peaks were generally broader when these compounds were injected as aqueous standards compared to those observed from plasma samples. The buffer or proteins in the plasma samples appeared aid in the chromatography of these amine-containing compounds. The analytes were retained more quantitatively and as a tighter band at the head of the ISRP column using plasma samples relative to aqueous

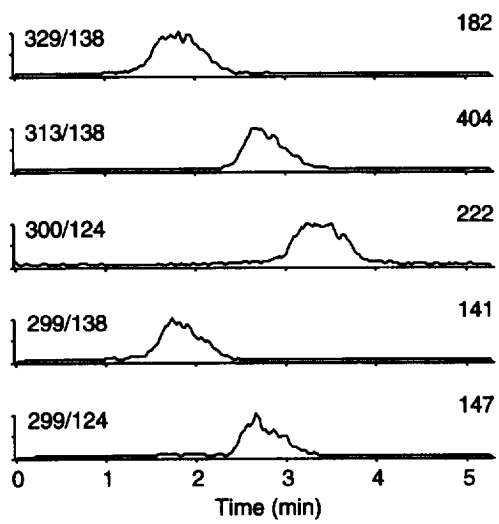


Fig. 5. Selected reaction monitoring chromatograms from injection of an aqueous standard solution containing 10 ng each of 7OH-G (*m/z* 329/138), granisetron (*m/z* 313/138), internal standard (*m/z* 300/124), metabolite B (*m/z* 299/138) and metabolite A (*m/z* 299/124).

standards. (We have observed this with a number of compounds.) As can be seen from the SRM chromatograms, 7OH-G and metabolite B co-eluted. Similarly, granisetron, metabolite A and I.S. co-eluted chromatographically. However, they were resolved by the mass spectrometer on the basis of their mass differences. Thus, none of the known metabolites of granisetron [35,36], which had been prepared synthetically, interfered with the analysis of either granisetron or 7OH-G. Typical SRM chromatograms of drug-free plasma and a plasma sample spiked with 0.1 ng/ml each of granisetron and 7OH-G are shown in Fig. 6. Based on the analysis of drug-free plasma samples, endogenous plasma components did not interfere with the analytes and the internal standard over the concentration range described here. Using 80 μl of plasma, the lower limit of quantification for granisetron and 7OH-G, was 0.05 ng/ml. Linear responses in analyte/internal standard peak-area ratios were observed for analyte concentrations ranging from 0.05 to 50 ng/ml of plasma. Correlation coefficients obtained using weighted ($1/y$) linear regression analysis of calibration lines were typically 0.997. The calibration lines were highly reproducible. The precision, as measured by the relative standard deviations at each of the spiked concentrations, and accuracy, evaluated by the average concentration back-calculated from the respective calibration lines, are shown in Table 2 for granisetron and 7OH-G.

3.4. Accuracy and precision

Results of a three-day validation study are displayed in Table 3 for both granisetron and 7OH-G. The intra-day precision of the method was indicated by the mean of the daily relative standard deviation (R.S.D). The inter-day precision of the method was indicated by the R.S.D. of the daily means. The mean accuracy of the method, as indicated by the ratio of the actual to theoretical concentrations, is also shown in Table 3.

3.5. Sample to sample carry over effects

Injection of drug free plasma samples immediately following plasma samples containing a high concentration of granisetron and 7OH-G (50 ng/ml)

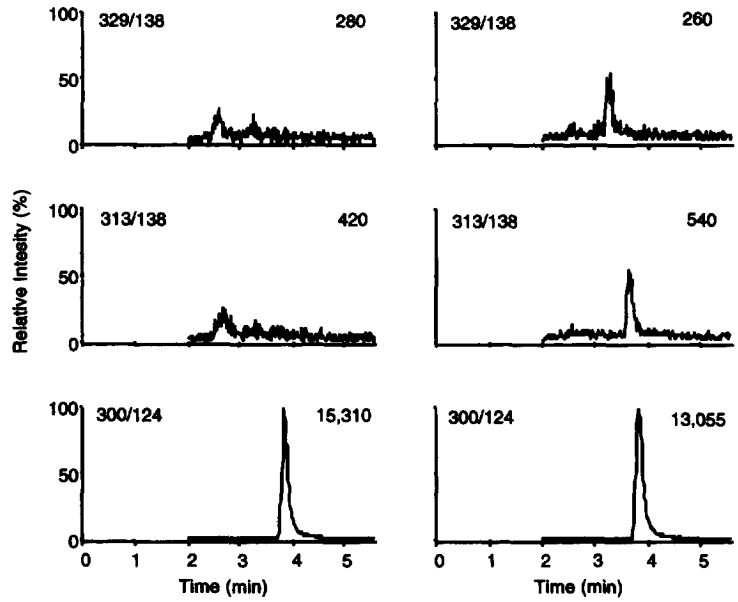


Fig. 6. Selected reaction monitoring chromatograms from blank dog plasma (left) spiked with internal standard (12.5 ng/ml, *m/z* 300/124) and dog plasma (right) spiked with 7OH-G (0.1 ng/ml, *m/z* 329/138), granisetron (0.1 ng/ml, *m/z* 313/138) and the internal standard (12.5 ng/ml, *m/z* 300/124).

resulted in a minor peak (0.05 ng/ml) in the granisetron SRM chromatograms, indicating slight carry over (0.1%) of drug from the previously injected sample. This carry-over of granisetron appeared to originate from the autosampler. Neither the ISRP guard columns nor the switching valves appeared to contribute to the observed carry-over. Use of longer wash and purge cycles and an appropriate wash solvent (depending the solubility of test analyte), can help minimize the carry-over. For granisetron, use of 25% aqueous acetonitrile solution to wash the autosampler yielded the best results.

4. Conclusions

We have described an on-line, automated sample preparation system coupled to LC–MS–MS and demonstrated its application for the analysis of

Table 2
Accuracy and precision data for granisetron and 7OH-G obtained from back calculated standard curve concentrations over four days

	Nominal concentration of granisetron and 7OH-G in dog plasma (ng/ml)									
	0.05	0.1	0.2	0.5	1.0	2.0	5.0	10.0	25.0	50.0
Granisetron										
Mean	0.051	0.108	0.210	0.556	1.099	2.186	5.257	10.435	26.446	47.391
S.D.	0.003	0.002	0.015	0.008	0.038	0.094	0.163	0.286	0.177	0.716
R.S.D. (%)	6.82	2.07	7.07	1.41	3.47	4.30	3.10	2.74	0.67	1.51
Accuracy	100.9	108.3	104.9	111.2	109.9	109.3	105.1	104.3	105.8	94.8
7OH-G										
Mean	0.051	0.109	0.208	0.509	1.044	2.132	5.333	10.889	27.498	54.168
S.D.	0.011	0.007	0.019	0.047	0.120	0.206	0.602	1.530	2.978	6.461
R.S.D. (%)	22.20	6.10	9.12	9.21	11.52	9.66	11.29	14.05	10.83	11.93
Accuracy	100.2	108.9	104.2	101.8	104.4	106.6	106.7	108.9	110.0	108.3

Table 3
Accuracy and precision data for granisetron and 7OH-G in dog plasma

Parameter	Concentration of granisetron and 7OH-G in dog plasma (ng/ml)							
	Granisetron				7OH-G			
	0.1	0.2	5.0	50.0	0.1	0.2	5.0	50.0
R.S.D. (%)								
Day 1	3.3	3.6	1.2	1.5	10.2	6.8	4.1	1.8
Day 2	4.8	3.8	0.7	1.5	5.4	5.0	1.7	4.0
Day 3	1.6	2.5	1.9	1.5	4.3	3.5	1.7	3.2
Error (%)[a]								
Day 1	2.5	−1.4	13.8	2.8	−1.3	−6.1	−3.3	−2.8
Day 2	−8.9	−11.3	−3.5	−13.4	−1.9	−8.8	−3.3	−8.3
Day 3	3.6	−9.5	5.6	−7.1	7.5	4.2	6.3	7.0
R.S.D. (%)								
Inter-day[b]	6.95	5.73	8.20	8.66	5.19	7.10	5.56	7.86
Intra-day[c]	3.24	3.30	1.30	1.50	6.63	5.06	2.50	2.99
Mean accuracy (%)	99.1	92.6	105.3	94.1	101.4	96.4	99.9	98.6

[a] (Calculated concentration−actual concentration)/actual concentration×100.
[b] Coefficients of variation of daily means.
[c] Mean of the daily coefficients of variation.

granisetron and its active 7-hydroxy metabolite in plasma. Using this method, the analytes were quantified accurately with high sensitivity (100 pg/ml) and precision. The on-line automated sample preparation system described here can be built easily in a very short time using inexpensive, commercially available components. The system has been very robust and has performed extremely well over the period of 4 months which it has been used. Using this on-line sample preparation approach, contamination or clogging of the HPLC, HPLC–MS interface, or source of the mass spectrometer was not a problem. The amount of routine cleaning required by the mass spectrometer source was equal to or less than that associated with samples prepared by off-line protein precipitation, or solid-phase or liquid–liquid extraction techniques. The ISRP columns have also proven to be robust and have been found to last for at least 60 to 75 injections (per cartridge). When the HPLC back pressure begins to increase, new guard cartridges can be installed and equilibrated quickly (less than 5 min) and no additional pretreatment of the cartridges has been necessary. In addition, the system has proven to be extremely versatile and reliable, and subsequently, has been used in the development of highly sensitive and fast LC–MS–MS methods, as well as LC–fluorescence and LC–UV methods, for several different types of compounds bearing a variety of functional groups.

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