



ELSEVIER

Journal of Chromatography A, 692 (1995) 195–202

JOURNAL OF
CHROMATOGRAPHY A

Simultaneous determination of granisetron and its 7-hydroxy metabolite in human plasma by reversed-phase high-performance liquid chromatography utilizing fluorescence and electrochemical detection

Venkata K. Boppana

Department of Drug Metabolism and Pharmacokinetics, SmithKline Beecham Pharmaceuticals, P.O. Box 1539, Mail Code UW 2711, King of Prussia, PA 19406-0939, USA

Abstract

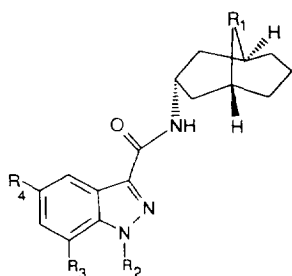
A highly sensitive and selective high-performance liquid chromatographic method was developed for the determination of granisetron and its active metabolite, 7-hydroxygranisetron (7OH-G) in human plasma. Granisetron is a selective 5-hydroxytryptamine receptor antagonist used in the treatment of cytotoxic drug-induced emesis. The method involves isolation of granisetron, 7OH-G and the internal standards from plasma by solid-phase extraction prior to reversed-phase ion-pair chromatographic separation on an octyl silica column with subsequent quantification of analytes simultaneously either with electrochemical (7OH-G) or fluorescence (granisetron) detectors which are placed in series. The recovery of granisetron and 7OH-G from human plasma was quantitative. Using 1 ml of plasma, the limits of quantification for granisetron and 7OH-G were 0.1 and 0.25 ng/ml, respectively. Linear responses in analyte/internal standard peak-area ratios were observed for analyte concentrations ranging from 0.1 to 50 ng/ml plasma. Precision and accuracy were within 13% across the calibration range for both granisetron and 7OH-G. The method was sufficiently sensitive, accurate and precise to support pharmacokinetic studies for granisetron and 7OH-G, in both normal and patient populations.

1. Introduction

Granisetron (BRL 43694, Fig. 1) {*endo*-1-methyl-N-(9-methyl-9-azabicyclo[3.3.1]non-3-yl)-1H-indazole-3-carboxamide} is a selective 5-hydroxytryptamine (5-HT₃) receptor antagonist which has been developed for the treatment of cytotoxic drug-induced emesis [1–3]. Initial studies in humans showed that, after intravenous and oral administration of [¹⁴C]granisetron, the drug was metabolized primarily to 7-hydroxygranisetron (7OH-G, BRL 55421), in addition to several other minor dealkylated metabolites and

hydroxy conjugates (Fig. 1). Since 7OH-G exhibited equivalent activity in both in vivo and in vitro pharmacologic models, its plasma concentrations needed to be quantitated along with granisetron in order to describe the overall pharmacokinetic and pharmacodynamic relationships following intravenous or oral administration of granisetron.

Although, an HPLC method [4] utilizing fluorescence detection was described for the quantification of granisetron in human plasma and urine, this method could not be adopted for the simultaneous quantitation of 7OH-G due to its



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
Metabolite C	N(=O)CH ₃	CH ₃	H	H
Metabolite E	N-CH ₃	CH ₃	H	OH

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

weak fluorescence response. This report describes a sensitive and specific HPLC method for the simultaneous determination of granisetron and 7OH-G in human plasma. The method involves isolation of granisetron and its hydroxy metabolite by solid phase extraction prior to reversed-phase ion-pair chromatographic separation on an octyl silica column with subsequent quantification of 7OH-G and granisetron with electrochemical and fluorescence detection, respectively.

2. Materials and methods

2.1. Chemicals

Granisetron hydrochloride (G), 7OH-G hydrochloride, metabolites A (BRL 46540), B (BRL 43110), C (BRL 46541), E (BRL 48397) and internal standard A (ISA, BRL 43704, Fig. 2) were supplied by SmithKline Beecham Pharmaceuticals (Worthing, West Sussex, UK). Internal standard B (ISB, SK & F 89124; 4-[2-(N,N-dipropylamino)ethyl]-7-hydroxy-2-(3H)-indolone · HCl, Fig. 2) was supplied by Drug Substances

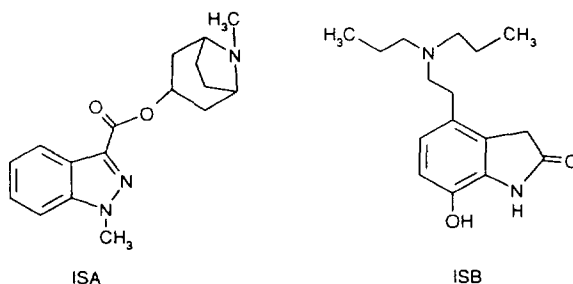


Fig. 2. Structures of internal standards.

and Products, SmithKline Beecham Pharmaceuticals (Swedeland, PA, USA). Glacial acetic acid was obtained from Mallinckrodt (Paris, KY, USA). Monobasic sodium phosphate, dibasic sodium phosphate, sodium acetate, HPLC-grade methanol and acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA). Disodium EDTA was obtained from EM Science (Cherry Hill, NJ, USA) and the sodium salt of hexanesulfonate was purchased from Regis (Morton Grove, IL, USA). C₂ silica solid-phase extraction (SPE) cartridges (1 ml) and the Vac-Elut manifold were purchased from Analytichem International (Harbor City, CA, USA).

2.2. Mobile phase

Sodium salt of hexanesulfonate (10 mM, 0.95 g) and 0.1 g of disodium EDTA were added to 405 ml of acetate buffer (0.1 M, pH 4.7). The solution was filtered first with a 0.5- μ m filter (type HA, Millipore). Acetonitrile (95 ml) was added to the above solution and the solution was sonicated under vacuum for 5 min.

2.3. Standard solutions

The stock standard solutions of granisetron, 7OH-G, ISA and ISB were prepared by dissolving appropriate amounts of the compounds in water to give a final solution 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, 1 and 0.1 μ g/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 solutions of ISA and ISB with water to give a solution concentration of 100 ng/ml. This solution was stable for 1 month when stored at 4°C.

2.4. Extraction of granisetron and 7OH-G from plasma

The C₂ extraction column was conditioned by successive washings with 1 ml of methanol, 1 ml of water and 1 ml of 1.0 M phosphate buffer, pH 7.0. An aliquot of heparinized plasma (1 ml) was transferred to a 75 × 12 mm polypropylene tube and mixed with 50 μl of water (containing standards when preparing standard curve), 50 μl of internal standard solution (100 ng/ml of each of ISA and ISB) and 500 μl of 1.0 M phosphate buffer, pH 7.0 (the phosphate buffer should be added to plasma just prior to application of sample onto the SPE column). The sample was applied to the C₂ column under reduced pressure at 3–4 in.Hg (1 in.Hg = 3386.38 Pa). Following sample application, the column was washed successively with 1 ml water and 1 ml of acetonitrile–water (40:60, v/v). The washing solvent was completely removed from the sorbent bed prior to elution. The column was then eluted first with 1 ml of methanol and then with 1 ml of 1% trifluoroacetic acid (TFA) methanolic solution. The combined eluate was collected into a 75 × 12 mm polypropylene tube. The eluate was evaporated under a gentle stream of nitrogen at 40°C and the residue was reconstituted in 200 μl of methanol–water (10:90, v/v). The sample was vortex mixed for approximately 30 s, centrifuged at approximately 2000 g for 5 min and transferred to an autosampler vial. The vials were centrifuged at approximately 2000 g for 5 min and 10–65 μl were injected into the HPLC system for analysis.

2.5. High-performance liquid chromatography

The isocratic HPLC system consisted of a Beckman (Palo Alto, CA, USA) Model 116 pump, an electrochemical detector (ESA, Brad-

ford, MA, USA) and a fluorescence detector (Model F1000; Hitachi, Danbury, CT, USA). Chromatographic separations were carried out on a 15 cm × 2.1 mm I.D. octyl silica column (Zorbax Rx C₈ column, Mac-Mod Analytical, Chadds Ford, PA, USA) which was preceded by a 3 cm × 2.1 mm I.D. C₈ guard column (ABI Instruments, Ramsey, NJ, USA). The column was maintained at 30°C and the mobile phase eluent, 0.1 M acetate pH 4.7 with 10 mM sodium hexanesulfonate and acetonitrile (405:95, v/v), was pumped at a flow-rate of 300 μl/min. Samples were injected using an HPLC auto-sampler (WISP Model 710B; Waters, Milford, MA, USA). The column eluent was first directed to the electrochemical cell and then to the fluorescence detector. The potentials at the electrochemical cell were maintained at 0.15 V (*E*₁, first cell) and 0.35 V (*E*₂, second cell) and the current was measured at *E*₂. An electrochemical guard cell with an applied potential of 0.4 V was also placed before the injector in order to eliminate any oxidizable impurities in the mobile phase and thus aid in reducing background current of the analytical cell. The excitation and emission wavelengths of the fluorescence detector were set at 305 and 360 nm, respectively. The chromatographic data from each detector were collected with an automated laboratory system (Nelson Chromatography, Cupertino, CA, USA).

2.6. Validation procedures

Four pools of plasma precision samples containing 0.1 (0.25), 0.25 (0.5), 5 (5), and 50 (50) ng/ml of granisetron (7OH-G) were prepared by adding appropriate volumes of standard solutions to drug-free plasma. These plasma samples were stored at –20°C until analysis was performed. Seven replicate samples from each pool were extracted and analyzed on three separate days. Concentrations were determined by comparison with a calibration curve prepared on the day of analysis. A weighted (1/*y*) linear regression was used to construct a calibration curve for the peak area ratio of analyte to internal standard vs. analyte concentration. From the data

obtained, inter-day precision [determined as relative standard deviations (R.S.D.s) of daily means], intra-day precision (determined as mean of the daily R.S.D.s) and mean accuracy were calculated.

3. Results and discussion

Quantitative methods described earlier for granisetron and its dealkylated metabolites are primarily based on the detection of native fluorescence of these compounds. Since 7OH-G lacks any appreciable fluorescence due to the electron-withdrawing nature of its phenolic group, alternate methods of detection were explored. The phenolic group present in 7OH-G was found to be easily oxidizable at low potential and offered an alternate method of detection for this compound utilizing an electrochemical detector. Since granisetron was not electrochemically active, both fluorescence and electrochemical detectors were employed in series following chromatographic separation of granisetron and 7OH-G for simultaneous quantification in a single chromatographic run. In essence, the method involves isolation of granisetron, its active metabolite 7OH-G and the internal standards (ISA and ISB) from plasma by SPE, followed by reversed-phase ion-pair chromatographic separation of the analytes on an octyl silica column with subsequent detection of electroactive analytes (7OH-G, ISB) and fluorescent analytes (granisetron, ISA) as described above. Careful optimization of chromatographic conditions and the use of HPLC columns of reduced internal diameter (2 mm) resulted in an on-column detection limit of 20 pg for granisetron and 50 pg for 7OH-G.

Fig. 3 displays fluorescence and electrochemical chromatograms obtained simultaneously from a single injection of an aqueous standard solution of granisetron, its metabolites (7OH-G, metabolite A, metabolite B, metabolite C and metabolite E) and the internal standards (ISA and ISB). As can be seen from these figures, the electrochemical chromatogram displays signals corresponding to 7OH-G, metabolite E and ISB with no response from other analytes, which

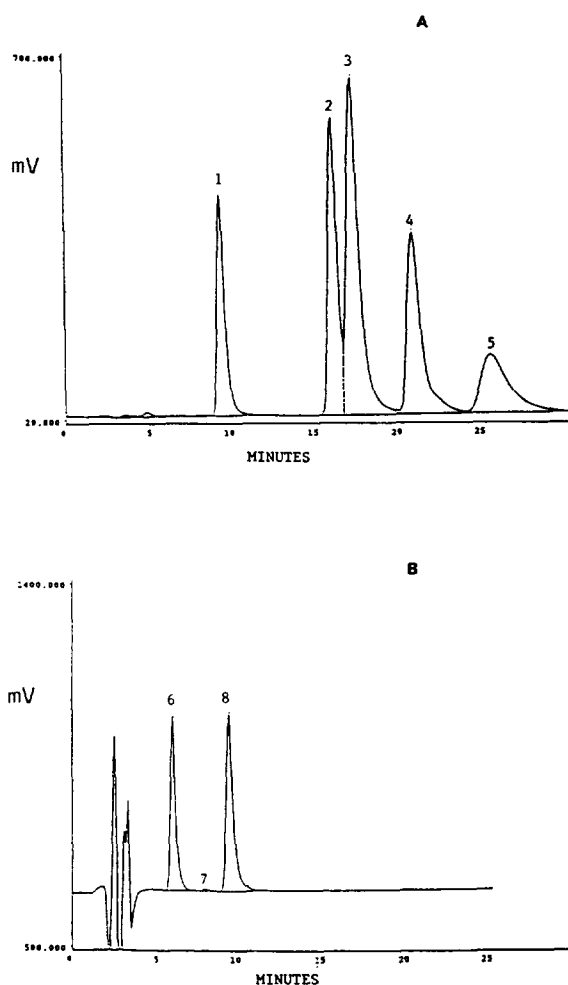


Fig. 3. Fluorescence (A) and electrochemical (B) chromatograms simultaneously obtained from a single injection of an aqueous standard solution of granisetron, its known metabolites and internal standards. Peaks: 1 = metabolite B; 2 = metabolite A; 3 = granisetron; 4 = ISA; 5 = metabolite C; 6 = ISB; 7 = metabolite E; 8 = 7OH-G.

were observed only in the fluorescence chromatogram. Although 7OH-G and metabolite B co-eluted chromatographically, 7OH-G was detected only by the electrochemical detector and metabolite B was detected only by the fluorescence detector.

3.1. Recovery and stability

The recovery of granisetron, 7OH-G and the internal standards from plasma was estimated

with seven determinations by comparing the peak areas obtained with processed samples to those obtained by direct injection of known amounts of the compounds equivalent to 100% recovery. The mean recovery of granisetron at plasma concentrations of 0.1 and 50 ng/ml was 107.9 ± 7.46 and 113.0 ± 3.16 , respectively. At plasma concentrations of 0.25 and 50 ng/ml, the mean recovery for 7OH-G was 97.7 ± 8.49 and 120.6 ± 7.11 , respectively. The mean recovery of ISA and ISB at a plasma concentration of 5 ng/ml was 123.7 ± 4.88 and 100.9 ± 5.14 , respectively.

The order in which methanol and TFA-methanol were applied to the C_2 SPE column to elute granisetron and 7OH-G greatly affected the recovery of these compounds. Quantitative recoveries of 7OH-G, granisetron, ISA and ISB were obtained when the SPE column was first eluted with methanol followed by TFA-methanol. Reversing the order of elution solvents resulted in poor recovery of 7OH-G. Granisetron, 7OH-G and the internal standards were stable in the final extracts in a sealed auto-sampler vial for at least 48 h following their isolation from plasma. However, repeat injection of the final extracts onto the HPLC system indicated marked degradation of 7OH-G with time, possibly due to oxidation of the analyte by the apparent entry of air through the septum of the vial during or after injection. Replacing the punctured septum with a new one minimized the degradation of 7OH-G and allowed the reanalysis of the samples.

3.2. Sensitivity, linearity and selectivity

Typical fluorescence and electrochemical chromatograms of plasma extracts obtained from drug-free human plasma and a plasma sample spiked with 0.1 ng/ml of granisetron and 0.25 ng/ml of 7OH-G are shown in Figs. 4 and 5, respectively. Based on the analysis of drug-free plasma samples, endogenous plasma components did not interfere with the analytes and the internal standards over the concentration range described here. In addition, none of the known metabolites of granisetron (metabolites A, B, C and E), which had been prepared synthetically,

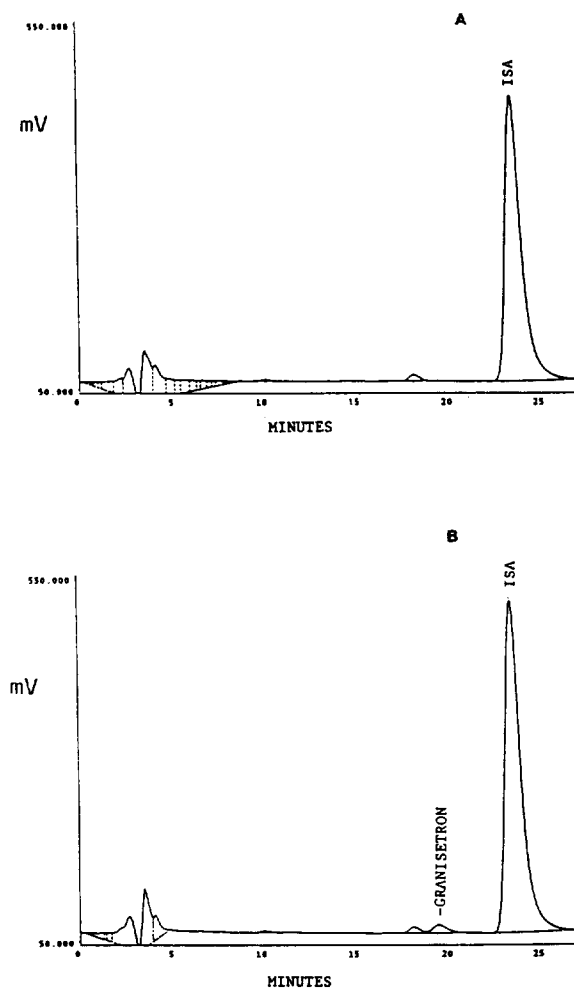


Fig. 4. Fluorescence chromatograms of extracts of drug-free human plasma spiked with ISA (A) and plasma spiked with 0.1 ng/ml of granisetron (B).

interfered with the analysis of either granisetron or 7OH-G. Fig. 6 displays fluorescence and electrochemical chromatograms obtained simultaneously from a single injection of extract obtained from a plasma sample drawn from a human volunteer 2 h after oral administration of 5 mg of granisetron. Using 1 ml of plasma, the limits of quantification for granisetron and 7OH-G were 0.1 and 0.25 ng/ml, respectively. Linear responses in analyte/internal standard peak-area ratios were observed for analyte concentrations ranging from 0.1 to 50 ng/ml plasma. Correla-

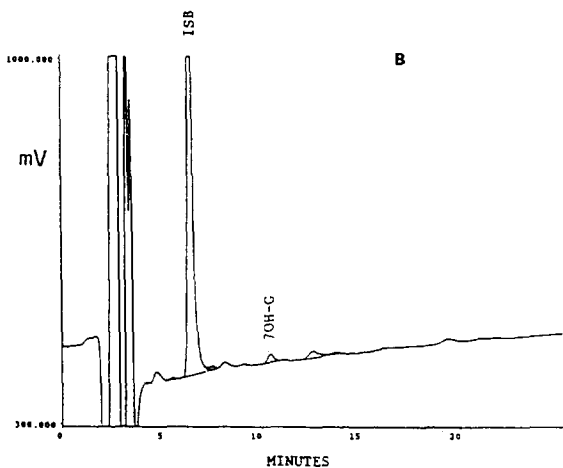
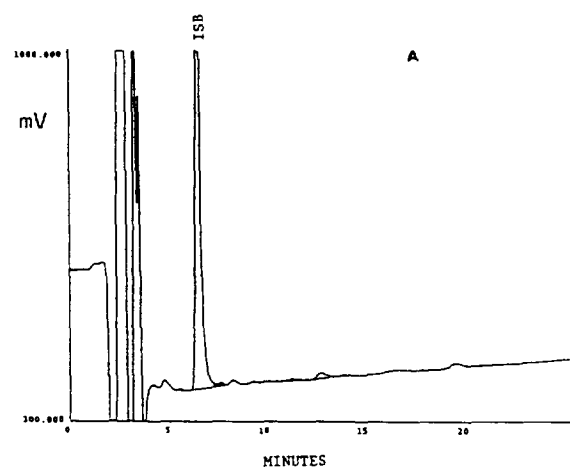


Fig. 5. Electrochemical chromatograms of extracts of drug-free human plasma spiked with ISB (A) and plasma spiked with 0.25 ng/ml of 7OH-G (B).

tion coefficients obtained using weighted ($1/y$) linear regression analysis of calibration curves were typically 0.999. The calibration curves 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 standard curves, are shown in Table 1 for granisetron and 7OH-G.

3.3. Accuracy and precision

Results of a three-day validation study are displayed in Table 2 for both granisetron and

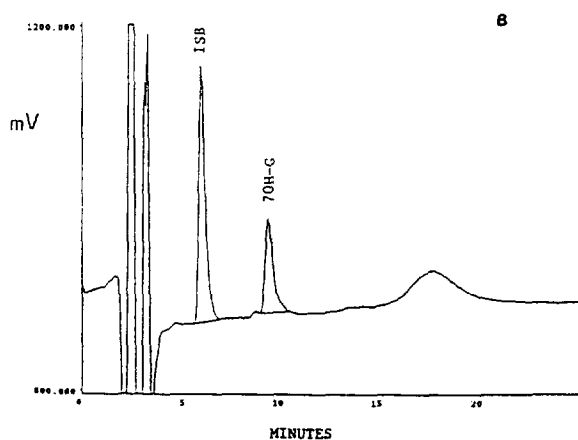
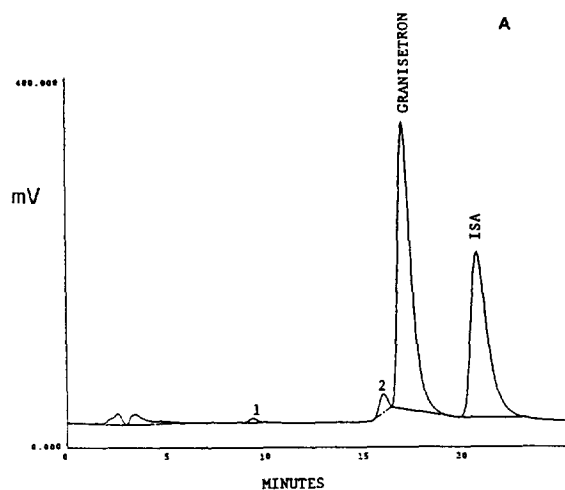


Fig. 6. Fluorescence (A) and electrochemical (B) chromatograms simultaneously obtained from a single injection of plasma extract from a human subject 2 h after oral administration of 5 mg of granisetron. The plasma concentrations of granisetron and 7OH-G are 5.82 and 3.81 ng/ml, respectively.

7OH-G. The intra-day precision of the method was indicated by the mean of the daily 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 2. The inter-day R.S.D.s of the method were also calculated by analyzing three pools of quality control plasma samples spiked with 0.5, 5.0 and 25 ng/ml of each of granisetron and 7OH-G over a period of

Table 1
Accuracy and precision data for granisetron and 7OH-G obtained from back-calculated standard curve concentrations over four days

	Nominal concentrations of granisetron and 7OH-G in plasma (ng/ml)								
	0.1	0.25	0.5	1.0	2.5	5.0	10.0	25.0	50.0
Granisetron									
Mean	0.101	0.261	0.51	1.027	2.559	5.186	10.269	24.834	49.683
S.D.	0.010	0.014	0.019	0.039	0.111	0.293	0.266	0.704	1.239
R.S.D. (%)	10.12	5.20	3.75	3.77	4.33	5.64	2.59	2.84	2.49
Accuracy (%)	101.0	104.4	102.0	102.7	102.4	103.7	102.7	99.3	99.4
7OH-G									
Mean	0.131	0.259	0.441	0.979	2.371	4.551	9.236	24.208	53.412
S.D.	0.028	0.031	0.037	0.127	0.277	0.274	0.768	1.086	2.626
R.S.D. (%)	21.02	11.969	8.37	12.97	11.68	6.02	8.31	4.49	4.92
Accuracy (%)	130.8	103.6	88.2	97.9	94.8	91.03	92.4	96.8	106.8

6 days. The inter-day R.S.D.s from the analysis of these samples, were found to be 7.32, 7.20 and 8.21% for granisetron ($n = 12$) and 12.92, 13.34 and 12.68% for 7OH-G ($n = 11$), respectively.

3.4. Application of the procedure to clinical plasma samples

The quantitative HPLC methodology described here provided for selective and sensitive

Table 2
Accuracy and precision data for granisetron and 7OH-G in plasma

Parameter	Concentrations of granisetron and 7OH-G in plasma (ng/ml)							
	Granisetron				7OH-G			
	0.1	0.25	5.0	50.0	0.25	0.5	5.0	50.0
R.S.D. (%)								
Day 1	7.03	6.59	2.20	2.74	9.10	12.49	9.22	5.13
Day 2	2.83	6.91	8.24	13.05	6.89	9.35	7.36	11.59
Day 3	7.03	2.99	0.91	9.26	9.68	9.19	6.46	1.92
Error (%)^a								
Day 1	+1.0	+2.8	+5.2	-4.2	-1.6	-1.8	-3.1	+1.4
Day 2	+2.0	+3.2	+5.2	+1.6	+4.0	-4.2	-5.6	+4.9
Day 3	0.0	-2.0	+0.3	+3.6	+9.2	-8.6	-5.0	-8.1
R.S.D. (%)								
Inter-day ^b	1.53	2.86	2.76	3.21	5.33	3.63	1.40	6.77
Intra-day ^c	5.63	5.50	3.78	8.35	8.56	10.34	7.68	6.21
Mean accuracy (%)	99.7	101.3	103.6	97.9	103.2	95.1	95.4	99.4

^a (Calculated concentration - actual concentration)/actual concentration \times 100.

^b R.S.D.s of daily means.

^c Mean of the daily R.S.D.s.

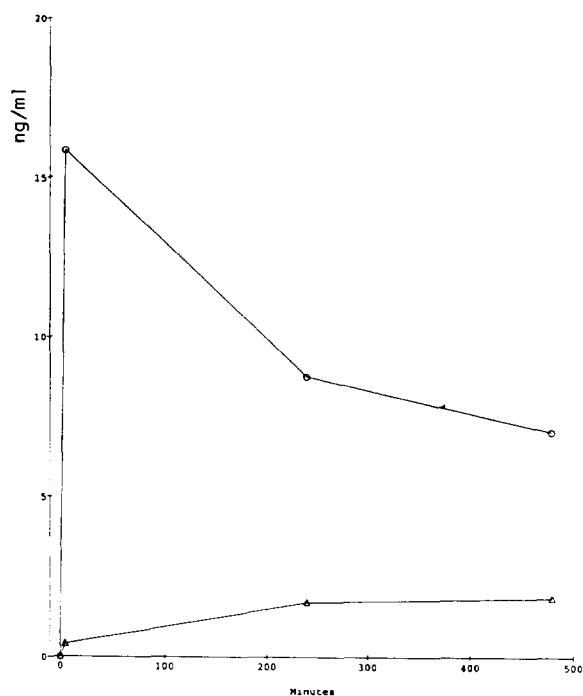


Fig. 7. Plasma concentrations of granisetron (○) and 7OH-G (△) following intravenous administration of 40 mg/kg of granisetron to a healthy volunteer.

detection of granisetron and 7OH-G in human plasma samples. Fig. 7 shows the application of this assay to a pharmacokinetic study in a human subject in which plasma concentrations of granisetron and 7OH-G were measured from serial blood samples drawn at 0, 5, 240 and 480 min following intravenous administration of 40 μ g/kg of granisetron. As can be observed from

the plasma concentration versus time curve, the methodology was sufficiently sensitive to support pharmacokinetic studies of granisetron in humans.

4. Conclusions

A sensitive HPLC method has been developed for the simultaneous determination of granisetron and its active metabolite, 7OH-G, in human plasma utilizing both fluorescence and electrochemical detection. The assay performed acceptably in a three-day validation over a concentration range of 0.1 to 50 ng/ml for granisetron and 0.25 to 50 ng/ml for 7OH-G. The method was sufficiently sensitive, accurate and precise to support pharmacokinetic studies for granisetron and 7OH-G in both normal and patient populations.

References

- [1] J. Carmichael, B.M.J. Cantwell, C.M. Edwards, W.G. Rapeport and A.L. Harris, *Br. Med. J.*, 297 (1988) 110.
- [2] J.W. Upward, B.D.C. Arnold, C. Link, D.M. Pierce, A. Allen and T.C.G. Tasker, *Eur. J. Cancer*, 26 (supp. 1) (1990) S12.
- [3] J. Carmichael, B.M.J. Cantwell, C.M. Edwards, B.D. Zussman, S. Thompson, W.G. Rapeport and A.L. Harris, *Cancer Chemother. Pharmacol.*, 24 (1989) 45.
- [4] A. Clarkson, P.E. Coates and B.D. Zussman, *Br. J. Clin. Pharmacol.*, 25 (1988) 136P.