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Short communication

Simultaneous determination of granisetron and 7-hydroxygranisetron in human plasma by high-performance liquid chromatography with fluorescence detection

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Abstract

A highly sensitive high-performance liquid chromatographic method for the determination of granisetron, a novel antiemetic agent for cancer therapy, and its pharmacologically active and major metabolite in human plasma is described. Methylation of the metabolite with trimethylsilyldiazomethane was employed for sample pretreatment. As a result of this treatment, the originally non-fluorescent metabolite could be detected simultaneously with granisetron using fluorescence detection. Both the compounds and the internal standard added were isocratically well resolved from endogenous peaks in plasma on an ODS column. The concentrations of the compounds were proportional to the peak-area ratios over the practical determination ranges of 0.2–100 ng/ml for granisetron and 0.1–50 ng/ml for the metabolite. In tests, reproducibilities for granisetron at 1 ng/ml and the metabolite at 0.5 ng/ml as determined by the relative standard deviation ($n = 3$) were less than 3.98 and 7.23%, respectively, in a single run.

1. Introduction

Granisetron, 1-methyl-N-(*endo*-9-methyl-9-azabicyclo[3.3.1]non-3-yl)-1*H*-indazole-3-carboxamide, is a selective 5-HT_3 receptor antagonist [1] discovered and developed by SmithKline Beecham Pharmaceuticals (Brentford, UK) as a potential therapeutic agent for the treatment of emesis induced by cytotoxic drugs for cancer such as cisplatin in humans. The agent is mainly metabolized in humans by aromatic hydroxylation to a pharmacologically active metabolite, 7-hydroxygranisetron (metabolite D), which is subsequently conjugated. A high-performance liquid chromatographic (HPLC) method [2] to determine granisetron had been developed previously. However, a method capable of detecting minute levels of metabolite D together with granisetron in human plasma was not available. In order to conduct future studies on the pharmacokinetics of the two compounds after oral

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administration to Japanese subjects, a simultaneous determination method was developed, involving HPLC after solid-phase extraction (SPE) and methylation of the metabolite to permit fluorescence detection based on the common main chemical structure.

2. Experimental

2.1. HPLC system

The chromatographic system consisted of an SCL-6B system controller, an LC-6A pump, a SIL-9A autoinjector, a CTO-6A column oven, an RF-535 fluorescence detector and a Chromatopac C-R4AX integrator (all from Simadzu, Kyoto, Japan). A Develosil ODS-5 (5 μm, 120 Å) column (25 cm × 4.6 mm I.D.) from Nomura Chemical (Seto, Japan) was used with a NewGuard RP-18 guard column (15 mm × 3.2 mm I.D.) from Applied Biosystems (San Jose, CA, USA) and was maintained at 45°C.

2.2. Reagents

Acetonitrile and methanol were of HPLC grade from Kokusan Chemical (Tokyo, Japan). The methylation reagent diazomethane (DAM) was prepared from N-methyl-N-nitroso-*p*-toluenesulfonamide (Aldrich, Milwaukee, WI, USA), dimethyl sulfate (DMS) was of extra-pure grade from Tokyo Kasei Kogyo (Tokyo, Japan) and trimethylsilyldiazomethane (TMSDM) was supplied as a 10% hexane solution by Chisso (Tokyo, Japan). N,N-Diisopropylethylamine (DIPEA) and tetra-*n*-butylammonium hydroxide solution (0.5 *M*) were purchased from Aldrich and Wako (Osaka, Japan), respectively. Water produced by a Milli-Q SP TOC system with a Labo Ionpure 12 apparatus (Japan Millipore, Tokyo, Japan) was used throughout. Phosphate buffer applied in the sample treatment procedure was prepared by dissolving 4.33 g of anhydrous disodium hydrogenphosphate and 3.04 g of sodium dihydrogenphosphate dihydrate (Wako) in 50 ml of water. Ammonium acetate buffer used in the eluent for HPLC was prepared by dissolving 15.4 g of ammonium acetate (Kanto Chemical, Tokyo, Japan) in *ca.* 1700 ml of water, adding 20 ml of tetra-*n*-butylammonium hydroxide, adjusting the pH to 4.70 with glacial acetic acid (Kanto Chemical) and diluting to 2000 ml with water.

2.3. Stock standard solutions

All reference standard materials depicted in Fig. 1 were synthesized and characterized by SmithKline Beecham Pharmaceutical Research Division (Welwyn Garden City, UK). Stock standard solutions of granisetron and metabolite D were prepared by accurately weighing about 10 mg (as free base) of each reference standard material and dissolving in 100 ml of water. A stock standard solution of the internal standard (I.S.), 2-methyl-N-(*endo*-9-methyl-9-azabicyclo[3.3.1]non-3-yl)-2*H*-indazole-3-carboxamide hydrochloride, was prepared in the same manner. The stock standard solutions were stored in amber-coloured silanized glass bottles at 4°C in the dark until required.

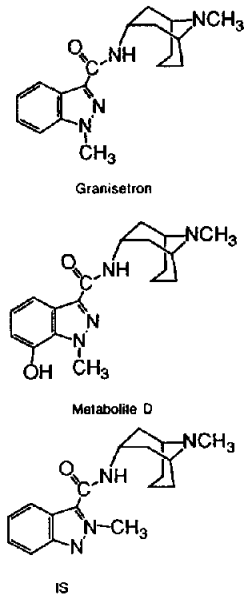

Fig. 1. Structures of granisetron, its major metabolite (metabolite D) and the internal standard (I.S.).

2.4. Calibration standard solutions

Working standard solutions for construction of the calibration graphs were prepared by diluting each stock standard solution with water to give the appropriate concentrations. Similarly, a 40 ng/ml I.S. working standard solution was prepared. Blank, drug-free plasma was obtained by centrifuging (*ca.* 1700 *g*) blood withdrawn from cubital veins of healthy Japanese volunteers into a Venojet tube containing EDTA disodium salt (VT-070NA; Terumo, Tokyo, Japan). The blank plasma was pooled and kept at *ca.* −70°C until used. To 1 ml of blank plasma, 100 μl of each working standard solution and I.S. working standard solution were added followed by 0.5 ml of 1 *M* phosphate buffer. The plasma solutions were subjected to the solid-phase extraction and methylation reaction pretreatment procedures as described in the next section.

2.5. Sample pretreatment

A plasma sample prepared as described above was applied to a Bond Elut C_2 cartridge column (100 mg of sorbent) (Varian, Harbor City, CA, USA) placed on a Vac-Elut SPS 24 (Varian) under reduced pressure (70–100 mmHg). The column was primed beforehand with 1 ml of methanol (twice), water (once) and 1 *M* phosphate buffer (pH 7.0) (twice). The loaded sample was washed with 1 ml of acetonitrile–water (2:3, v/v) and the washing solvent was removed from the sorbent bed prior to elution. All compounds of interest were eluted with 800 μl of methanol into a borosilicate glass tube with a screw-cap (Iwaki Glass, Tokyo, Japan). The eluate was evaporated to dryness under a stream of nitrogen at 50°C. To the residue, 30 μl each of TMSDM and DIPEA solutions in methanol were added and the tube was tightly capped and then heated at 50°C for 20 min. After cooling to room temperature, the solution was evaporated to dryness under the same conditions as above. The residue was reconstituted in 300 μl of 10% aqueous methanol and centrifuged (*ca.* 1700 *g*) for 5 min, then a 150-μl aliquot of the supernatant was subjected to HPLC analysis with fluorescence detection ($\lambda_{ex.}$ 310 nm, $\lambda_{em.}$ 420 nm).

3. Results and discussion

3.1. Sample pretreatment

As liquid–liquid extraction was found not to be practical for achieving efficient extraction of granisetron and metabolite D, a procedure utilizing SPE was developed. In this approach, the use of an I.S. was considered to be necessary to achieve accurate and reproducible quantification. This was because several steps in manual procedures such as evaporation and reconstitution needed to be applied in SPE and also in the following methylation reaction. In addition, it is useful even if the nature of sorbent beds varies between manufacturing lots. In tests, Bond Elut C_{18} and C_2 sufficiently retained the both compounds as well as I.S. employed and C_2 was selected because all the compounds of interest eluted from the sorbent bed with a smaller volume of methanol with much less co-elution of endogenous contaminants. For washing, acetonitrile–water (2:3, v/v) showed the maximum capability to remove endogenous compounds with no significant loss of the retained compounds. In addition, conditioning the sorbent bed twice with 1 ml of 1 *M* phosphate buffer (pH 7.0) before sample application was found to be important to achieve reproducible retention of the compounds.

Among structural analogues of granisetron, those having a hydroxyl indazole structure, including metabolite D, were non-fluorescent, indicating that the dissociation of the phenol-type hydroxyl group led to quenching of fluorescence. Labelling the hydroxyl moiety with an electron-donating reagent can theoretically prevent dissociation, thus restoring the characteristic fluorescence of the indazole structure. Methylation of metabolite D was considered to be a simple and suitable approach to obtain a method for the simultaneous determination of metabolite D and granisetron. This was achievable because the wavelengths of excitation and emission for both

granisetron and methylated metabolite D are close enough for the same detection conditions to be employed. Initially, the methylation reagents DAM, DMS and TMSDM were examined. DAM was difficult to handle and degradation occurred with DMS owing to the basic conditions required for the reaction. TMSDM appeared to be a stable and safe substitute for DAM [3]. The methylation conditions for metabolite D were optimized using TMSDM in the presence of methanol and DIPEA as the recommended catalyst for a selective reaction [4]. A concentration of metabolite D of 20 ng/ml was selected for the derivatization conditions.

The required amount of methanol for the reaction was first examined with 1% TMSDM in hexane–methanol solution by changing the proportion of methanol in hexane. As demonstrated in Fig. 2, the proportion of methanol considerably affected the methylation reaction, and 20–60% (v/v) of methanol gave similar high yields. In addition to methanol, acetonitrile, acetone, tetrahydrofuran, methyl ethyl ketone, ethyl acetate, chloroform and diisopropyl ether were examined, but none of them increased the reaction yield. Therefore, methanol was added at 50% (v/v) to the reaction solution for further examination.

The TMSDM content in the solution was varied from 0.5 to 5% (v/v). The peak area of metabolite D increased with increasing TMSDM concentration up to 2% (v/v) and then levelled off. For subsequent measurements the 5% (v/v) of TMSDM was selected. The requirements for DIPEA as a catalyst was then confirmed and 1% (v/v) of the compound was found to be sufficient for the reaction.

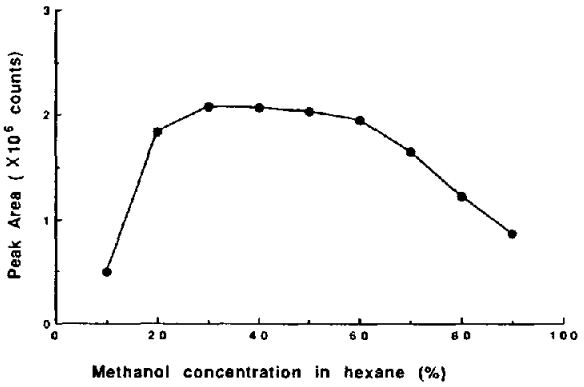

Fig. 2. Effect of methanol content on the methylation reaction. Chromatographic conditions are described in the text. TMSDM concentration, 5% (v/v); reaction temperature, 50°C; reaction time, 20 min.

The final methylation solution was prepared to contain 5% (v/v) TMSDM, 1% (v/v) DIPEA and 50% (v/v) methanol in hexane. A 50-μl volume methylation solution was sufficient for the reaction and larger volumes gave no substantial increase in peak area of the methylated metabolite D.

The reaction temperature was next examined, as methylation at ambient temperature has sometimes been reported to require as long as 15 h [3]. In our hands, gentle heating was found to be effective in shortening the reaction time without adverse reactions. In tests, no significant difference in peak areas was observed after 10-min incubation at 40, 50 and 60°C and the peak areas neither increased nor deteriorated with incubation times up to 60 min. Accordingly, reaction at 50°C for 15 min was employed in subsequent work.

Using the above conditions, the effects of plasma constituents were studied by comparing the chromatograms for several sets of standard solutions, derivatized and extracted, and with those obtained after adding the same amounts to plasma. Close agreement of the peak-area ratios was obtained over the concentration range tested, as depicted in Fig. 3, indicating that the established reaction conditions were applicable to authentic plasma samples. In addition, the methylation reaction was confirmed not to affect the determination of granisetron.

3.2. HPLC conditions

As demonstrated in Fig. 4, granisetron, metabolite D and the I.S. were isocratically well resolved from the peaks of endogenous substances on a Develosil ODS column with the eluent consisting of 0.1 *M* ammonium acetate buffer (pH 4.7) containing 1% (v/v) of tetra-*n*-butylammonium hydroxide and methanol in the ratio 7:3 (v/v). The flow-rate was maintained at

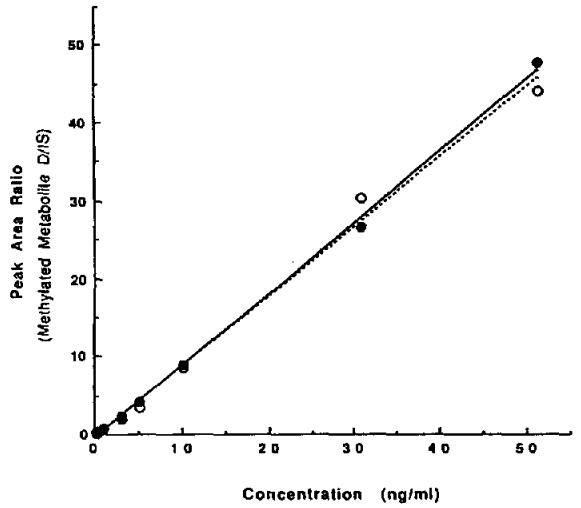

Fig. 3. Effect of plasma constituents on the established methylation reaction. (○). Standard sample of metabolite D chiratly methylated; (●) standard sample of metabolyte D added to plasma and methylated after the extraction procedure.

1.0 ml/min. Fluorescence detection was achieved by tuning the excitation and emission maxima for the methyl derivative of metabolite D at 310 nm for excitation and 420 nm for emission, which were slightly different from those for granisetron reported previously [2,5]. This was because the plasma concentration of metabolite D was presumed to be much lower than that of granisetron.

3.3. Quantification

The calibration graphs for granisetron and metabolite D were linear ($r > 0.999$) over the practical determination ranges, 0.2–100 and 0.1–50 ng/ml, respectively, with an injection volume of 150 μl. The detection limit of granisetron was 42 pg/ml and that of metabolite D was 27 pg/ml at a signal-to-noise ratio of 3. Five-day repetitive assays of plasma samples spiked with the standards at two concentration levels gave acceptable precision and reproducibility (Table 1), indicating a reliable method for practical assays. Correlation of the concentrations of granisetron determined by the conventional (x) [2] and the present methods (y) was satisfactory ($y = 1.056x$, $r = 0.997$) over the concentration range 0–60 ng/ml.

4. Conclusions

Precolumn derivatisation of metabolite D with TMSDM gave a methylated product with sufficient fluorescence to allow simultaneous determination with granisetron under the same chro-

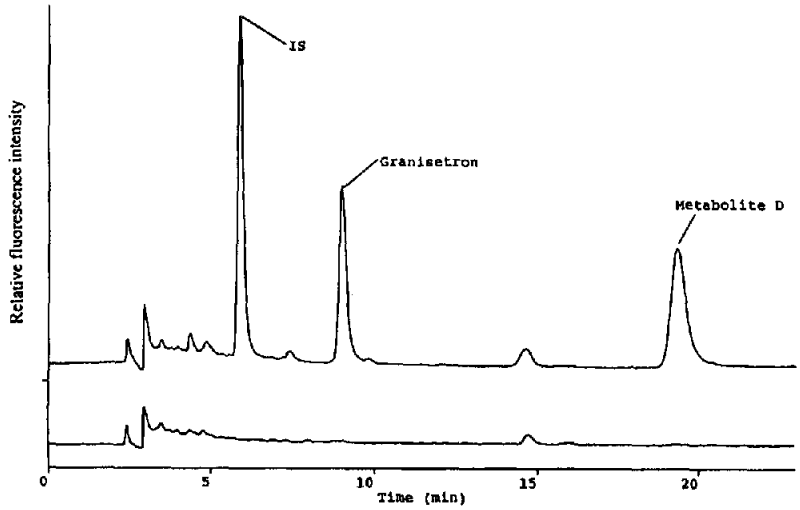

Fig. 4. Representative chromatograms of (a) plasma spiked with the I.S., granisetron and metabolite D and (b) blank plasma. Chromatographic conditions are described in the text.

Table 1
Accuracy and reproducibility of the proposed method for granisetron and metabolite D

Added (ng/ml)	Concentration found[a] (ng/ml)				
	Day 1	Day 2	Day 3	Day 4	Day 5
Granisetron					
1.03	1.05 ± 0.04 (3.98)	1.07 ± 0.03 (2.34)	1.08 ± 0.02 (1.93)	1.13 ± 0.03 (2.71)	1.04 ± 0.02 (2.00)
20.53	21.02 ± 0.16 (0.76)	20.85 ± 0.43 (2.05)	21.03 ± 0.08 (0.36)	20.40 ± 0.06 (0.30)	20.83 ± 0.43 (2.08)
Metabolite D					
0.51	0.52 ± 0.04 (7.23)	0.51 ± 0.02 (2.98)	0.53 ± 0.02 (2.86)	0.55 ± 0.02 (2.76)	0.52 ± 0.03 (4.87)
10.26	10.94 ± 0.24 (2.19)	10.50 ± 0.56 (5.31)	10.69 ± 0.12 (1.08)	10.41 ± 0.16 (1.52)	10.64 ± 0.70 (6.55)

[a] Mean ± S.D. ($n = 3$), with R.S.D. (%) in parentheses.

matographic conditions. The assay method was shown to be sensitive and accurate to apply to analysis of plasma samples from Phase I pharmacokinetic studies in Japanese subjects.

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