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High-performance liquid chromatographic determination of granisetron in human plasma

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Abstract

This paper describes a high-performance liquid chromatographic method (HPLC) with fluorometric detection for the analysis of granisetron in plasma. The detection is performed at 305 nm for excitation and 365 nm for emission. The method involves sample clean-up by liquid–liquid extraction. N-(1-Naphthyl) ethylenediamine dihydrochloride is used as internal standard. Toluene and phosphate buffer were added to 0.5 ml of plasma added to the internal standard. After extraction, the organic layer was separated and then evaporated to dryness. The residue was reconstituted in eluent mixture. An aliquot was injected onto the HPLC column, Spherisorb CN, equilibrated with an eluent mixture constituted by acetonitrile–phosphate buffer (pH 4.5) (15:85). The proposed technique is reproducible, selective, reliable, and sensitive. Linear detector responses were observed for the calibration curve standards in the range of 0.50 to 100 ng/ml. Extraction recovery from plasma proved to be more than 90%. Precision expressed as C.V. was in the range 2 to 8%. As low as 0.3 ng of granisetron per ml of plasma can be measured with good accuracy. The method has been validated, and stability tests under various conditions have been performed. Its sensitivity is adequate for pharmacokinetic studies.

Keywords: Granisetron

1. Introduction

Intravenous drug therapy in a patient with cancer often requires the co-administration of several medications. Severe nausea and vomiting are frequently associated with many cytotoxic chemotherapeutic agents and are a major cause of distress to the patient [1]. Granisetron (BRL 43694A), [endo-N-(9-methyl-9-azabicyclo(3,3,1)non-3-yl)-1-ethyl-1H-indazole-3-carboxamide] HCl (I) (Fig. 1) is a serotonine type 5-HT_3 receptor antagonist which is effective in preventing chemotherapy-induced emesis following intravenous or oral administration [2–6]. The efficacy and safety of the intravenous formulation of granisetron have been examined in an intensive clinical research program involving over 3000 patients undergoing chemotherapy for

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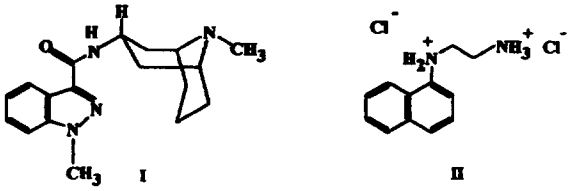

Fig. 1. Structural formulae of granisetron (I) and internal standard (II) used for the analytical method.

malignant disease. These studies demonstrated that a single intravenous dose of 40 μg/kg given immediately prior to chemotherapy completely prevented the onset of nausea and vomiting in approximately 60% of patients receiving high-dose cisplatin regimens [4] and as many as 80% of patients undergoing treatment with other emetogenic regimens [4]. In all studies, granisetron was extremely well tolerated, with headache (in around 15% of patients) being the most common adverse event considered probably attributable to the drug. Unlike metoclopramide, granisetron has no dopamine-blocking properties and hence does not carry the potential to induce extrapyramidal symptoms. Although granisetron has been shown to be effective when given alone, the addition of dexamethasone or methylprednisolone to the regimen has resulted in improved effectiveness [10]. To date, few published methods [11–13] are available in the literature. Some of them involved solid-phase extraction prior to reversed-phase ion-pair chromatography [12,13].

This paper describes a specific, reliable, and sensitive analytical method based on reversed-phase liquid chromatography for the quantitation of granisetron in plasma. The sample preparation involved sample clean-up by liquid–liquid extraction. This method was validated according to validation procedures, parameters and acceptance criteria based on USP XXIII guidelines and recommendations of Shah et al. [14,15]. The low quantitation limit (0.3 ng/ml) was achieved by a combination of a selective extraction procedure and efficient chromatography. This method has enhanced precision due to the inclusion of an internal standard. The simple sample preparation also makes the method appropriate for analysis of the large number of samples obtained in pharmacokinetic studies in patients undergoing cytotoxic chemotherapy for malignant disease.

2. Experimental

2.1. Materials and reagents

Granisetron or [endo-N-(9-methyl-9-azabicyclo(3,3,1)non-3-yl)-1-ethyl-1H-indazole-3-carboxamide] HCl (I) was obtained from SmithKline Beecham (Crawley, UK). The internal standard (II), N-(1-naphthyl) ethylenediamine dihydrochloride, was obtained from Sigma (St Louis, MO, USA) (Fig. 1). Acetonitrile and toluene were LiChrosolv grade (Merck, Darmstadt, Germany) and used without further purification. Sodium dihydrogen phosphate (Prolabo, Paris, France), sodium hydroxide and orthophosphoric acid (Merck) were all analytical grade.

Orthophosphoric acid 10% (v/v) and 0.1 *M* sodium hydroxide were prepared in purified water (Aguettant, Lyon, France). The buffer (pH 4.5) consisted of 0.1 *M* sodium dihydrogen phosphate in purified water adjusted to pH 4.5 with orthophosphoric acid. The buffer (pH 12) consisted of 0.1 *M* sodium dihydrogen phosphate in purified water adjusted to pH 12 with sodium hydroxide.

Stock solutions of I and II (1 mg/ml) were prepared in purified water. Working solutions of I were prepared in purified water by diluting the stock solution 10 and 100 fold, extemporaneously.

Pooled free plasma samples from healthy volunteers were used for validation.

2.2. Instrumentation

The isocratic system consisted of the following components: a Shimadzu Model LC9A pump and a Shimadzu Model RF-535 variable-wavelength fluorescence detector (Kyoto, Japan), and a Rheodyne loading valve fitted with a 20-μl sample loop (Touzart & Matignon, Paris, France). Data were sampled and analysed on a

Shimadzu Model C-R5A integrator (chart speed, 0.5 mm/min).

2.3. Chromatographic conditions

Isocratic separation was achieved using a Spherisorb CN stainless-steel column (250 × 4.6 mm I.D., 10 μm packing) supplied by Touzart & Matignon.

The mobile phase, containing 15 parts acetonitrile and 85 parts phosphate buffer (pH 4.5) was de-aerated ultrasonically prior to use. The flow-rate was 2 ml/min, which corresponds to a pressure of about 187 bar. Chromatography was performed at ambient temperature. The analytes were detected at 305 nm for excitation and 365 nm for emission.

2.4. Extraction procedure

Plasma sample (0.5 ml) was pipetted in a 5-ml glass centrifuge tube. Internal standard solution (7 μg) was added. The mixture was vortex-mixed for 10 s, then 1.5 ml toluene and 0.25 ml phosphate buffer (pH 12) were added, and the tubes were shaken for 20 min. The sample was then centrifuged at 3000 *g* for 10 min to separate the layers. One millilitre of the supernatant was transferred into a 5-ml glass tube and evaporated to dryness under nitrogen at 37°C. The residue was reconstituted with 40 μl of eluent by vortex-mixing for 10 s prior to injection, then 20 μl were injected into the column.

2.5. Instrument calibration

Calibration standards for control plasma were prepared using concentrations of 0.5, 1, 10, 25, 50 and 100 ng/ml. The volume added was always smaller than or equal to 2% of the total volume of the samples, so that the integrity of the plasma was maintained.

2.6. Data analysis

The ratio of the peak area of I to that of II was used as the assay parameter. Peak-area ratios were plotted against theoretical concentrations.

Standard calibration curves were obtained from unweighted least-squares linear regression analysis of the data.

The linearity of the method was confirmed using the classical statistical tests, that is, comparison of intercept with zero and correlation coefficient.

2.7. Specificity

To evaluate the specificity of the method, 0.5 ml of drug-free plasma was carried through the assay procedure, and the retention times of endogenous compounds in plasma were compared with those of granisetron and internal standard.

The interference from the main metabolite of granisetron (7-hydroxygranisetron) was checked.

Interference from other drugs that could be co-administered was also studied using different plasma samples from hospitalised patients assayed according to the extraction procedure and chromatographic conditions described above; consequently, the possible interference with metabolites of these drugs was also checked. The following drugs were tested: dexamethasone, methyl prednisolone, ondansetron, cimetidine, anthracyclines, 5-fluorouracil, methotrexate, *cis*-dichlorodiaminoplatinum, etoposide.

2.8. Recovery

The extraction efficiency (recovery) of compound I was determined by comparing peak areas from drug-free plasma spiked with known amounts of drug (10, 25, 50 and 100 ng/ml) assayed accordingly versus peak areas of the same concentrations prepared in mobile phase injected directly onto the analytical column. Each sample was determined in replicate ($n = 4$).

In order to study the co-extracted biological material effect, recoveries were also computed by comparison of extracts from spiked samples with blank extracts spiked after extraction.

The extraction efficiency was also determined for the internal standard.

2.9. Precision and accuracy

Between-day repeatability was assessed by performing replicate analyses of spiked samples at concentrations of 1, 20, 45 and 220 ng/ml against a calibration curve. The procedure was repeated for different days ($n = 6$) on the same spiked standards.

Within-day repeatability was determined by treating the same spiked plasma samples in replicate ($n = 4$) the same day.

The accuracy, expressed as percent deviation of observed concentration from theoretical concentration, with the relative error was evaluated.

2.10. Determination of the limit of quantitation and limit of detection

The limit of quantitation (LOQ) was determined from the peak and the standard deviation of the noise level, S_N. The LOQ was defined as the sample concentration resulting in a peak area of ten times S_N. The estimate of S_N was determined by extrapolation to zero. To determine the analytical error on the LOQ, spiked plasma was used. The limit of detection (LOD) was defined as the sample concentration resulting in a peak area of three times S_N.

2.11. Stability studies

In previously published data, the stability of granisetron in aqueous solutions (0.05, 0.15 and 1 mg/ml) was studied over three days at room temperature, seven days at +4°C, and one month at −20°C [16]. The long-term freezer stability in plasma at −20°C has been also evaluated during a two-month period for concentrations of 1, 5, 10, 20 and 50 ng/ml [17].

In the present study, the stability of granisetron in plasma was assessed from spiked samples (5 and 50 ng/ml), after bench-top storage at room temperature, at +4°C and at −20°C, over 96 h. They were analyzed immediately after preparation and after storage. Prior to the analysis of samples after cold storage, they were brought to room temperature (20°C) and vortex-mixed well. Each determination was performed in duplicate.

3. Results

3.1. Retention times

Observed retention times were 6.64 and 3.60 min for I and II, respectively. The capacity factors were 8.49 for I and 4.14 for II (Fig. 2).

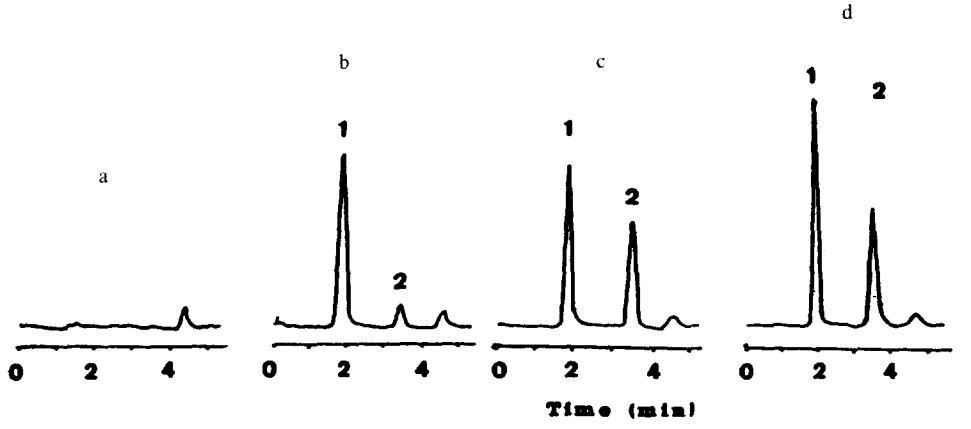

Fig. 2. Chromatograms of blank plasma (a) and of plasma spiked with 10 (b) and 50 ng/ml (c) of granisetron. Chromatogram of unknown plasma sample (d) from a patient treated intravenously with 3 mg of granisetron, sample collected 20 min after administration. Concentration found: 41 ng/ml. Peak 1 is the internal standard, and peak 2 is granisetron. For chromatographic conditions see text.

The resolution between the two compounds was 16.2, and the selectivity was 2.05.

3.2. Specificity

A representative chromatogram is shown in Fig. 2. No peaks interfered at the retention times of I or II. No interference was found with 7-hydroxygranisetron and with all drugs tested that could be co-administered with granisetron.

3.3. Linearity

In plasma the peak-area ratio of I over the internal standard varied linearly with concentration over the range used, which was 0.5 to 100 ng/ml. The correlation coefficients for calibration curves were equal to or better than 0.998.

Intra-assay reproducibility was determined for calibration curves prepared the same day in replicate ($n = 6$) using the same stock solutions. The intra-day average slope of the fitted straight lines was $0.0124 \pm 4.76 \cdot 10^{-4}$ ng^{-1} ml (C.V. = 3.84%), the mean intercept of the calibration curves was $-4.19 \cdot 10^{-3}$. The corresponding mean (±S.D.) coefficient of the linear regression analysis was $0.999 \pm 1.14 \cdot 10^{-3}$ (C.V. = 0.114%).

For calibration curves prepared on different days ($n = 12$), the mean results were as follows: slope = $0.0127 \pm 6.30 \cdot 10^{-4}$ ng^{-1} ml (C.V. = 4.96%), coefficient of the linear regression analysis = $0.9993 \pm 8.28 \cdot 10^{-4}$ (C.V. = 0.083%) and intercept = $-4.07 \cdot 10^{-3}$.

For each point of calibration standards, the concentrations were recalculated from the equation of the linear regression curves (experimental concentrations). Intra-day and inter-day variabilities at concentrations of calibration standards are given in Table 1.

The linearity of this method was statistically confirmed. For each calibration curve, the intercept was not statistically different from zero.

3.4. Recovery

The mean recovery of I, computed by comparison of extracts from spiked samples with the same concentrations prepared in mobile phase, averaged 95.8 ± 6.7% ($n = 16$). This did not vary statistically over the range of concentrations studied.

No effect of the co-extracted biological material was detected; recoveries computed by comparison of extracts from spiked samples with blank extracts spiked after extraction averaged 96.5 ± 1.26% ($n = 6$).

Table 1
Inter- and intra-assay reproducibilities of the HPLC analysis

Theoretical concentration (ng/ml)	Experimental concentration (mean ± S.D.) (ng/ml)	C.V. (%)
Inter-assay reproducibility (n = 12)		
0.5	0.515 ± 0.0339	6.58
1	1.00 ± 0.0619	6.19
10	9.77 ± 0.274	2.80
25	24.8 ± 0.405	1.63
50	50.4 ± 0.943	1.87
100	99.3 ± 2.30	2.32
Intra-assay reproducibility (n = 6)		
0.5	0.529 ± 0.0436	8.25
1	1.02 ± 0.0826	8.10
10	9.70 ± 0.338	3.48
25	24.2 ± 1.50	6.20
50	51.5 ± 2.70	5.24
100	100.2 ± 2.43	2.42

The mean recovery of II averaged 72.3 ± 0.03% ($n = 3$).

3.5. Precision and accuracy

For concentrations of calibration standards ranging from 0.5 to 100 ng/ml, the precision around the mean value did not exceed 8.5% C.V. (Table 1). This precision was 13% for a concentration of 0.3 ng/ml.

Within-run and between-run precision of the method were assessed by analysing spiked samples prepared in human plasma at different concentrations, in replicate, the same day, and on different days. The results for accuracy, within-day and between-day repeatabilities are presented in Table 2.

3.6. Limit of quantitation and limit of detection

The limit of quantitation was 0.3 ng/ml. At this level, the analytical error was less than 15%. The limit of detection was 0.1 ng/ml with a precision of 28%.

3.7. Stability studies

In aqueous solutions, granisetron was stable over three days at 20°C, seven days at +4°C and thirty days at −20°C. No significant deviation was found from the nominal values [16].

In plasma, granisetron was stable when frozen at −20°C for two months; the relative error from nominal values was less than 10% from 5 to 50 ng/ml and averaged 16% at 1 ng/ml [17].

In the present study, we have determined the stability of granisetron in plasma during a 96-h period at room temperature, at +4°C and at −20°C. At all temperatures tested, no significant difference appeared between concentrations of granisetron after storage and nominal values. The mean recovery was 99.9 ± 1.68% (98 to 101.1%) for 5 ng/ml and 100.8 ± 0.69% (100 to 101.6%) for 50 ng/ml.

4. Discussion and conclusion

The present HPLC method involves a rapid assay for the determination of granisetron in plasma with a run time of 8 min. Assay performance was assessed both on the basis of the statistical characteristics of individual calibration lines and from the results of quality control samples. This method has been validated for concentrations ranging from 0.3 to 100 ng/ml that spans what is currently thought to be the clinically relevant range for granisetron concentrations in body fluids. This technique has a good reproducibility, accuracy, and sensitivity. The separation between granisetron and endogenous substances was satisfactory. Moreover, the

Table 2
Accuracy and precision of the HPLC method

Theoretical concentration (ng/ml)	Experimental concentration (mean ± S.D.) (ng/ml)	C.V. (%)	Deviation from theoretical value (%)	Recovery (%)
Within-day ($n = 4$)				
1	0.95 ± 0.081	8.52	5.0	95.0
20	21.2 ± 0.360	1.70	6.0	106
45	46.1 ± 4.04	8.76	2.44	102
220	212.9 ± 11.9	5.60	3.2	96.7
Between-day ($n = 6$)				
1	1.01 ± 0.0764	7.57	1.0	101
20	20.4 ± 0.521	2.55	2.0	102
45	47.0 ± 2.60	5.53	4.4	104
220	226.6 ± 7.60	3.34	3.0	103

specificity from drugs that may be co-administered is good. Due to its weak fluorescence response, the simultaneous quantitation of 7-hydroxygranisetron, which represents 4–6% of the administered dose in plasma [13], was not possible.

Stability studies carried out directly in plasma indicated that samples were stable for at least two months when stored at −20°C.

The method described was found to be suitable for the analysis of all samples collected during pharmacokinetic investigations in humans.

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