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Simultaneous determination of omeprazole and 5′-hydroxyomeprazole in human plasma by liquid chromatography–tandem mass spectrometry

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Abstract

A method for the simultaneous determination of omeprazole and 5′-hydroxyomeprazole in human plasma is described. Isolation of the analytes from plasma was achieved via solid-phase extraction using a polymeric sorbent based cartridge. The analytes were chromatographed under reversed-phase conditions on a Zorbax XDB-C_8 column (50×4.6 mm). The HPLC mobile phase consisted of a mixture of acetonitrile–water (21:79, v/v) containing 10 m*M* ammonium hydroxide. The apparent pH of the mobile phase was adjusted to 8.5 with formic acid prior to use. A Sciex API III+ tandem mass spectrometer equipped with a heated nebulizer atmospheric pressure chemical ionization interface was used as a detector and was operated in the positive ion mode. Multiple reaction monitoring using the precursor→product ion combinations of *m*/*z* 362→214, 346→198 and 316→147 was used to detect 5′-hydroxyomeprazole, omeprazole and internal standard, respectively. The method was validated in the concentration range of 10–500 ng/ml plasma with adequate assay precision and accuracy. The assay was used to determine the cytochrome P450 2C19 phenotype of subjects participating in clinical trials of compounds under development.

Keywords: Omeprazole; Hydroxyomeprazole

1. Introduction

Wide variations in pharmacokinetic parameters, such as half-life, are often observed within study populations during the development of drug candidates. One possible explanation for this inter-subject variability relates to the different metabolic capacities of the study subjects. The cytochrome P450 enzyme system is known to play a major role in the metabolism of many drug entities. This enzyme system is composed of a family of enzyme isoforms, several of which are known to exhibit genetic polymorphisms. Individuals may be classified as possessing either extensive or poor metabolism phenotypes as a result of the genetic heterogeneity of the cytochrome P450 system; poor metabolizers have a diminished metabolic capacity as compared to individuals who have the extensive metabolism phenotype.

The CYP2C19 isoform of cytochrome P450 is known to exhibit genetic polymorphism. Therefore, variations in pharmacokinetic parameters observed in subjects participating in clinical studies of compounds suspected of being metabolized by this enzyme may be rationalized by knowledge of the metabolic phenotype of the study subjects. The metabolic behavior of probe drugs known to be metabolized by specific enzymes is frequently used to assign metabolic phenotypes. In the case of

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CYP2C19, the hydroxylation of omeprazole, as defined by the ratio of omeprazole to 5′-hydroxy-omeprazole present in samples obtained 2 h after dosing, has recently been proposed as a method to assign the metabolic phenotype of this enzyme [1]. An assay to simultaneously quantitate omeprazole and 5′-hydroxyomeprazole is thus needed to apply this method of metabolic phenotyping.

While several methods for the determination of omeprazole and 5′-omeprazole have been described in the literature [2–4], these assays suffer from inadequate sensitivity and the use of a complex automated sample preparation system [3], long analysis times [4], or the use of toxic halogenated solvents during sample preparation [2,4]. A simultaneous assay for the determination of omeprazole and 5′-hydroxyomeprazole using solid-phase extraction (SPE) for analyte isolation and high-performance liquid chromatography (HPLC) with tandem mass spectrometry (MS–MS) is the subject of this paper.

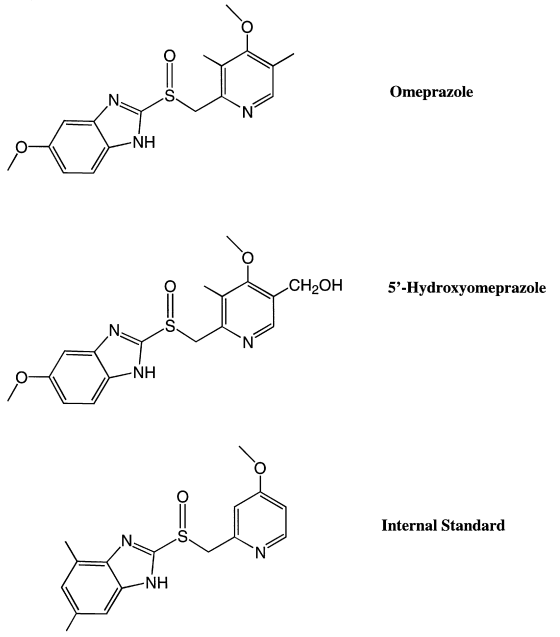

Fig. 1. Structures of omeprazole, 5′-hydroxyomeprazole and the internal standard

2. Experimental

2.1. Materials

Omeprazole and the internal standard (Fig. 1) were obtained from the Chemical Data Department of Merck Research Labs. (Rahway, NJ, USA). The reference standard of 5′-hydroxyomeprazole (Fig. 1) was provided by Astra Hässle (Molndal, Sweden). Solvents (HPLC grade) were from EM Science (Gibbstown, NJ, USA). Oasis SPE columns (60 mg sorbent bed) were purchased from Waters (Milford, MA, USA). Drug-free human plasma was obtained from Sera-Tech Biologicals (New Brunswick, NJ, USA). All other reagents were A.C.S. grade and were used as received.

2.2. Instrumentation

The HPLC system consisted of a Perkin-Elmer (Norwalk, CT, USA) Model 250 pump, a Waters WISP 715 autosampler, and an API III$^+$ triple quadrupole tandem mass spectrometer (PE-Sciex, Thornhill, Canada) equipped with a heated nebulizer atmospheric pressure chemical ionization (APCI) interface.

2.3. Chromatographic conditions

The HPLC mobile phase consisted of a mixture of acetonitrile–water (21:79, v/v), to which ammonium hydroxide was added to give a final concentration of 10 m*M* in the total mobile phase volume. The apparent pH of the mobile phase was then adjusted to 8.5 with formic acid. The mobile phase was filtered through a nylon membrane (0.20 μm) prior to use. Mobile phase was delivered at a flow-rate of 1 ml/min through a Zorbax (Mac-Mod Scientific, Chadds Ford, PA, USA) XDB-C_8 column (3 μm silica, 50×4.6 mm). The sample injection volume was 35 μl, while the total run time was 11 min.

2.4. Mass spectrometric conditions

The mass spectrometer was interfaced to the HPLC system via a heated nebulizer probe that was maintained at 500°C. Nebulizer (nitrogen) pressure was set at 80 p.s.i. (1 p.s.i.=6894.76 Pa). Nebulizer and curtain gas (nitrogen) flows were set at 1.4 and 0.6 l/min, respectively. Positive chemical ionization was effected by a corona discharge needle (+3.0

μA) and the sampling orifice potential was set at +30 V. The first quadrupole, Q1, was set to monitor the protonated molecules $(M+H)^+$ at m/z 362, 346 and 316 for 5′-hydroxyomeprazole, omeprazole and internal standard, respectively. Product ions resulting from collision-induced fragmentation at Q2 (argon as collision gas at $260 \cdot 10^{12}$ atoms cm^{-2}) were monitored via Q3 at m/z 214 (5′-hydroxyomeprazole), 198 (omeprazole) and 147 (internal standard). The electron multiplier setting was −4.0 kV and detector electronics were set to counts of ten.

2.5. Data acquisition and analysis

Data acquisition and analysis were performed using RAD and MacQuan software (PE-Sciex). Unknown sample concentrations were calculated from the equation $y=mx+b$, as determined by the weighted $(1/y^2)$ linear least-squares regression of the calibration line constructed from the peak area ratios of analyte to internal standard versus analyte concentration.

2.6. Preparation of standards

Stock solutions (100 μg/ml) of omeprazole and 5′-hydroxyomeprazole were prepared by weighing 1 mg of each compound into separate 10-ml volumetric flasks, dissolving the materials in 2 ml of methanol and filling the flasks to volume with 0.1 *M* phosphate buffer (pH 11). A stock solution containing omeprazole and 5′-hydroxyomeprazole, each at a concentration of 40 μg/ml, was prepared by pipetting 4 ml of each of the 100 μg/ml solutions into a 10-ml volumetric flask and filling the flask to volume with the pH 11 phosphate buffer. A stock solution containing the analytes, each at a concentration of 4 μg/ml, was prepared by diluting 1 ml of the 40 μg/ml stock solution to 10 ml with phosphate buffer, pH 11.

Working standards of 10, 8, 4 and 2 μg/ml were prepared by dilution of the 40 μg/ml stock solution, while working standards of 1, 0.4 and 0.2 μg/ml were prepared by dilution of the 4.0 μg/ml stock with phosphate buffer, pH 11.

Analysis standards were prepared by adding 50 μl of each working standard to 1 ml of drug-free plasma contained within a disposable glass culture tube. The resulting standards were utilized to quantitate clinical plasma samples ranging in concentration from 10 to 500 ng/ml.

2.7. Sample preparation procedure

A 1-ml volume of the clinical plasma sample was pipetted into a disposable glass culture tube. To this tube was added 50 μl of 0.1 *M* phosphate buffer, pH 11. A 50-μl aliquot of internal standard solution (2 μg/ml), followed by a 0.5 ml aliquot of 0.5 *M* phosphate buffer, pH 7.5 were then added to the tubes containing the samples and analysis standards. The tubes were mixed by vortexing. Oasis 60 mg SPE columns were conditioned by passing, using a vacuum manifold, 1 ml of methanol, followed by 1 ml of water through the columns. The plasma samples or standards were applied, under vacuum, to the columns. The columns were washed with 1 ml of a 15% solution of methanol in water. Following the wash step, the columns were removed from the vacuum manifold and suspended within 125×16 mm disposable glass tubes. Three ml of methanol was added to the columns. The methanol was then allowed to flow through the columns using gravity in order to elute the analytes. The methanol was evaporated under a stream of nitrogen and the samples were reconstituted in 700 μl of mobile phase. In order to remove particulate matter, the reconstituted samples were passed through Centrex 0.45 μm centrifugal filters prior to transfer into autosampler vials. A 35-μl aliquot of each filtered, reconstituted sample was injected into the LC–MS–MS system for analysis.

3. Results

3.1. Assay selectivity

Figs. 2–4 show chromatograms of extracted control plasma, a plasma standard and a plasma sample taken from a subject following the administration of 20 mg omeprazole, respectively. A comparison of Fig. 2 with Figs. 3 and 4 illustrates that no endogenous peaks co-elute with any of the analytes. The selectivity of the method is further illustrated by the fact that all pre-dose plasma samples from

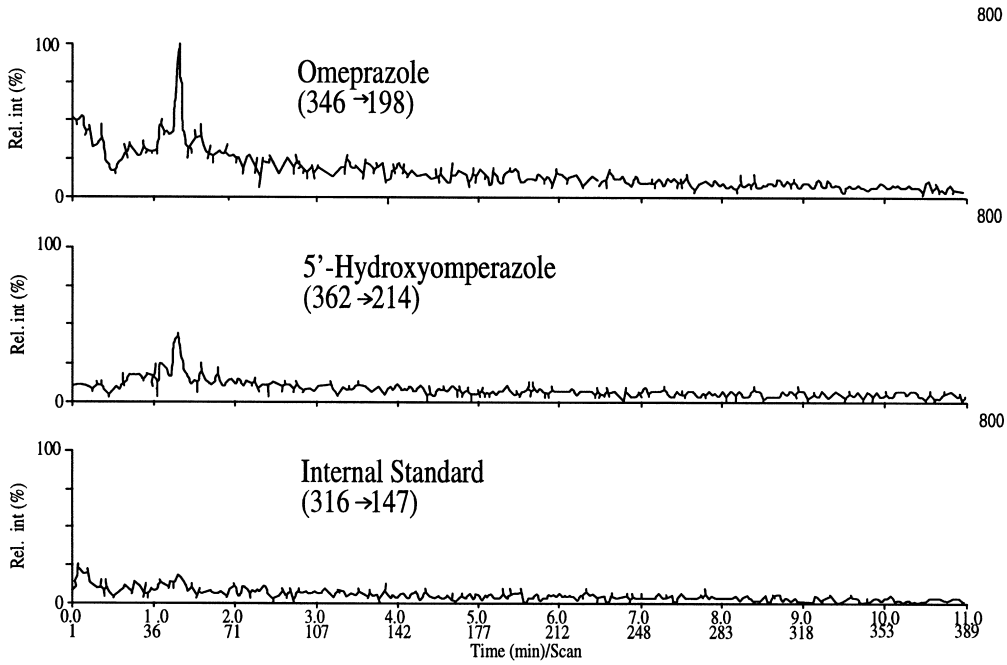

Fig. 2. Chromatogram of control plasma. The number in the upper right hand corner of each chromatogram represents peak height expressed in arbitrary units.

subjects involved in clinical trials were free of interfering peaks.

3.2. Linearity

Weighted (weighting factor $=1/y^2$ where y=peak area ratio) least-squares regression calibration curves constructed by plotting the peak area ratio of omeprazole or 5′-hydroxyomeprazole to internal standard vs. standard concentration yielded coefficients of regression typically greater than 0.99 over the concentration range of 10 to 500 ng/ml for each analyte. The use of the weighted least-squares regression resulted in less than a 10% deviation between the nominal standard concentrations and the experimentally determined standard concentrations calculated from the regression equations.

3.3. Extraction recovery

The efficiency of the extraction procedure was evaluated by comparing the instrument response of standards spiked into extracts of control plasma with that of extracted standards. The extraction efficiency evaluation was conducted at all points on the calibration curve. The mean recoveries of the analytes over the range of the standard curve were found to be 104.3% and 100.3% for 5′-hydroxyomeprazole and omeprazole, respectively. The extraction efficiency for the internal standard, spiked into plasma at a concentration of 100 ng/ml, was 92.0%.

3.4. Assay precision and accuracy

Replicate standards (n=5) were analyzed to assess the within-day variability of the assay. The mean assayed concentrations as well as the mean accuracy and relative standard deviations (R.S.D.s) of the analyses are shown in Table 1.

Quality control (QC) samples containing each of the analytes at concentrations of 25 and 375 ng/ml in plasma were prepared and frozen (−20°C) in 1 ml volumes. Following initial replicate (n=5) within-day analysis, the quality controls were analyzed three times over a one-week period to assess the inter-day variability of the assay. The results (Table 2) indi-

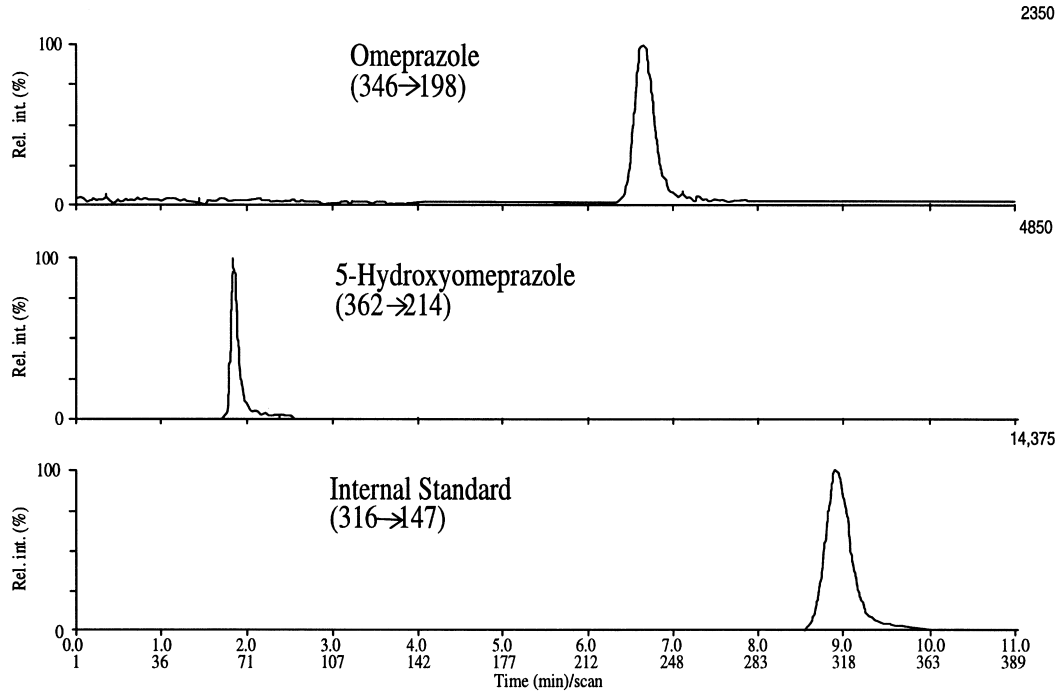

Fig. 3. Chromatogram of a plasma standard containing omeprazole (10 ng/ml) and 5′-hydroxyomeprazole (10 ng/ml) with internal standard added at a concentration of 100 ng/ml. The number in the upper right hand corner of each chromatogram represents peak height expressed in arbitrary units.

cate that the inter-day variability of the assays, as measured by the R.S.D. is under 4%. The results also indicate that frozen plasma samples containing omeprazole and 5′-hydroxyomeprazole appear stable for at least one week when stored frozen at −20°C.

3.5. Limit of quantification

The limit of quantification of the assay, defined as the lowest concentration that yielded an within-day R.S.D. of less than 10% and a within-day accuracy of between 90 and 110% of nominal concentration was 10 ng/ml.

4. Discussion

Several methods for the determination of omeprazole and 5′-hydroxyomeprazole have been described in the literature [2–4]. These methods, however, suffer from limitations such as the use of a time consuming liquid–liquid extraction procedure with toxic halogenated solvents (methylene chloride) for sample preparation [2,4], complex automated extraction systems [3], and the need to utilize separate extraction and chromatography systems for each analyte [2]. In recent years, the use of LC–MS–MS has been found to be suitable for the analysis of analytes in biological fluids [5–7]. A number of examples from our laboratories demonstrating the applicability of this method for the quantitative determination of various drug candidates have been described [8–10]. It was felt that the use of LC–MS–MS in combination with SPE for sample preparation would have the potential to overcome the limitations associated with the existing methods for the determination of omeprazole and 5′-hydroxyomeprazole.

Full-scan positive-ion spectra of omeprazole and 5′-hydroxyomeprazole yielded predominantly the protonated molecular ions at m/z 346 and 362, respectively. The product ion mass spectra of these protonated molecular ions (Fig. 5) show the presence of one predominant product ion for each compound

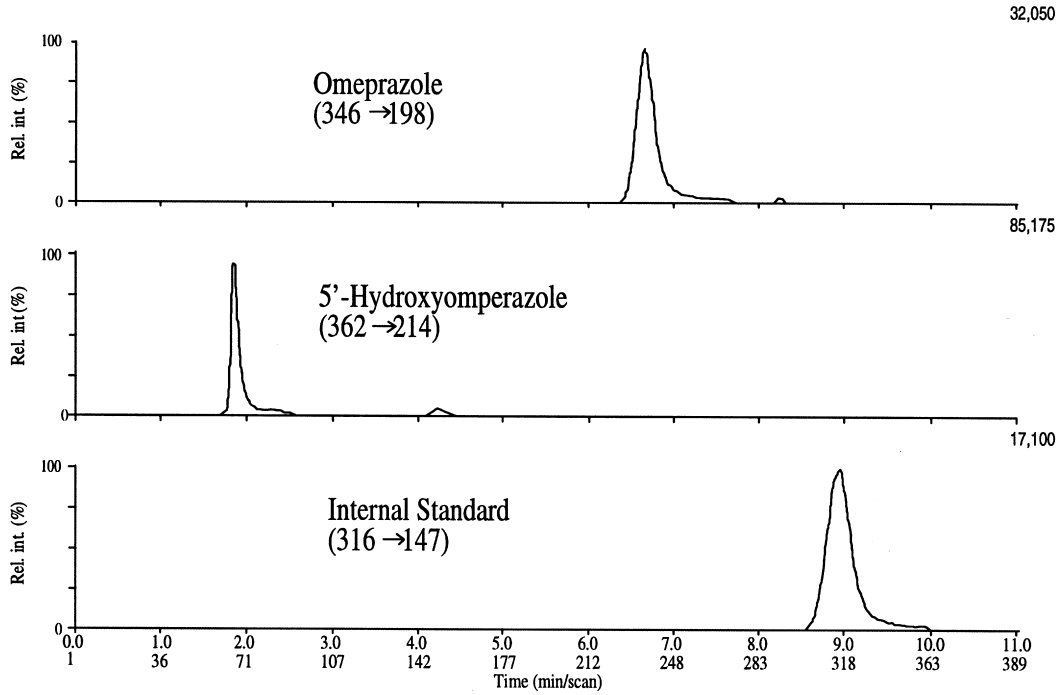

Fig. 4. Chromatogram of a plasma sample obtained from a subject 2 h after the oral administration of 20 mg omeprazole. The concentrations of omeprazole and 5′-hydroxyomeprazole in this sample were found to be equivalent to 106.9 and 166.0 ng/ml, respectively. Internal standard was added to the sample at a concentration of 100 ng/ml. The number in the upper right hand corner of each chromatogram represents peak height expressed in arbitrary units.

at m/z 198 and m/z 214 for omeprazole and 5′-hydroxyomeprazole, respectively. These product ions probably result from the loss of the benzimidazole portions of the molecules.

Development of a suitable chromatographic system was required following the mass spectral characterization of the analytes. Omeprazole, when prepared in solutions with a pH of less than 8, has been

Table 1
Intra-day precision and accuracy data for the determination of omeprazole and 5′-hydroxyomeprazole in plasma as assessed by the replicate (n=5) analysis of standards

Nominal standard concentration (ng/ml)		Mean (n=5) analyzed standard concentration (ng/ml)		Accuracy[a] (%)		Precision[b] (%)	
Omeprazole	5-Hydroxyomeprazole	Omeprazole	5-Hydroxyomeprazole	Omeprazole	5-Hydroxyomeprazole	Omeprazole	5-Hydroxyomeprazole
10.0	10.0	10.1	10.2	100.9	102.3	4.7	2.8
20.0	20.0	19.9	19.2	99.4	95.9	4.0	6.0
50.0	50.0	49.3	50.0	98.6	99.9	2.0	2.5
100.0	100.0	98.2	99.9	98.2	99.9	1.8	5.7
200.0	200.0	202.2	206.3	101.1	103.1	2.8	5.8
400.0	400.0	408.4	401.5	102.1	100.4	2.9	4.3
500.0	500.0	505.1	510.2	101.0	102.0	2.1	6.7

[a] Expressed as [(mean observed concentration)/nominal concentration)]×100.
[b] R.S.D..

Table 2
Initial within-day analysis of quality control (QC) samples and inter-day variability of the assay as assessed by R.S.D.s of low and high QC samples

	QC concentration (ng/ml)			
	5′-Hydroxyomeprazole		Omeprazole	
Nominal concentration:	25	375	25	375
Initial mean (n=5) assayed concentration	25.7	386.3	26.2	391.7
S.D.[a]	0.63	25.1	0.78	11.7
R.S.D. (%)	2.5	6.5	3.0	3.0
Daily Results[b]				
	25.2	379.3	25.7	372.6
	23.9	358.0	25.9	368.4
	24.9	360.4	26.1	373.4
Mean	24.7	365.9	25.9	371.5
S.D.[a] (n=6)	0.74	11.3	0.27	4.9
R.S.D. (%)	3.0	3.1	1.1	1.3

[a] Standard deviation.
[b] Three runs over a one week period. Average of two values for each concentration.

reported to degrade rapidly [11]. Therefore, in order to maximize analyte stability, a chromatographic system that utilized a mobile phase with a pH greater than 8 was required. Such a mobile phase necessitated the use of an alkali-resistant HPLC stationary phase. Kobayashi et al. [4] utilized a polymer-coated C_{18} column (Capcell Pak C_{18}) to chromatograph omeprazole and its metabolites under alkaline conditions. Attempts to use this column with a volatile, LC–MS–MS suitable, mobile phase consisting of acetonitrile, water, ammonium hydroxide and formic acid resulted in rapid degradation of peak shape. Zorbax XDB columns have been reported to be suitable for use with mobile phases whose pH is greater than 7 [12]. Substitution of a Zorbax XDB column for the Capcell Pak column eliminated the column deterioration problem; no loss in column performance was observed with the XDB column in two weeks of operation. The stability of the analytes under these chromatographic conditions was assessed based on the repetitive injection, over a 24-h period, of a solution of the analytes prepared in mobile phase. No change in instrument response was observed during this time, hence, it was concluded that the analytes were stable under the chromatographic conditions of the assay.

The simultaneous isolation of omeprazole and its 5′-hydroxy metabolite presented a challenge due to the wide difference in the polarity of the molecules. The methylene chloride–phosphate buffer distribution ratio of 5′-hydroxyomeprazole has been reported to be almost two-orders of magnitude less than that of omeprazole [2]. Hence, while the quantitative recovery of omeprazole from 1 ml of plasma was achieved using 1 ml of methylene chloride for extraction, high extraction recovery of 5′-hydroxyomeprazole required the use of 10 ml of methylene chloride per 1 ml of plasma [2]. We desired to develop a SPE procedure as an alternative to liquid–liquid extraction for sample preparation in order to minimize the exposure of laboratory personnel to large volumes of toxic halogenated organic solvents.

Recently, Oasis, a polymer based SPE sorbent has become commercially available [13]. This sorbent is claimed to retain molecules that differ widely in their polarities, making it a logical choice to evaluate with respect to the SPE of 5′-hydroxyomeprazole and omeprazole. Analyte recovery from 1 ml of plasma was found to be essentially quantitative when a SPE column containing 60 mg of sorbent was employed for the extraction together with methanol (3 ml) as the elution solvent. Elution of the columns was performed by suspending them in culture tubes and allowing the methanol to flow through the columns using only the force of gravity. Use of a vacuum manifold for the elution step was found to reduce

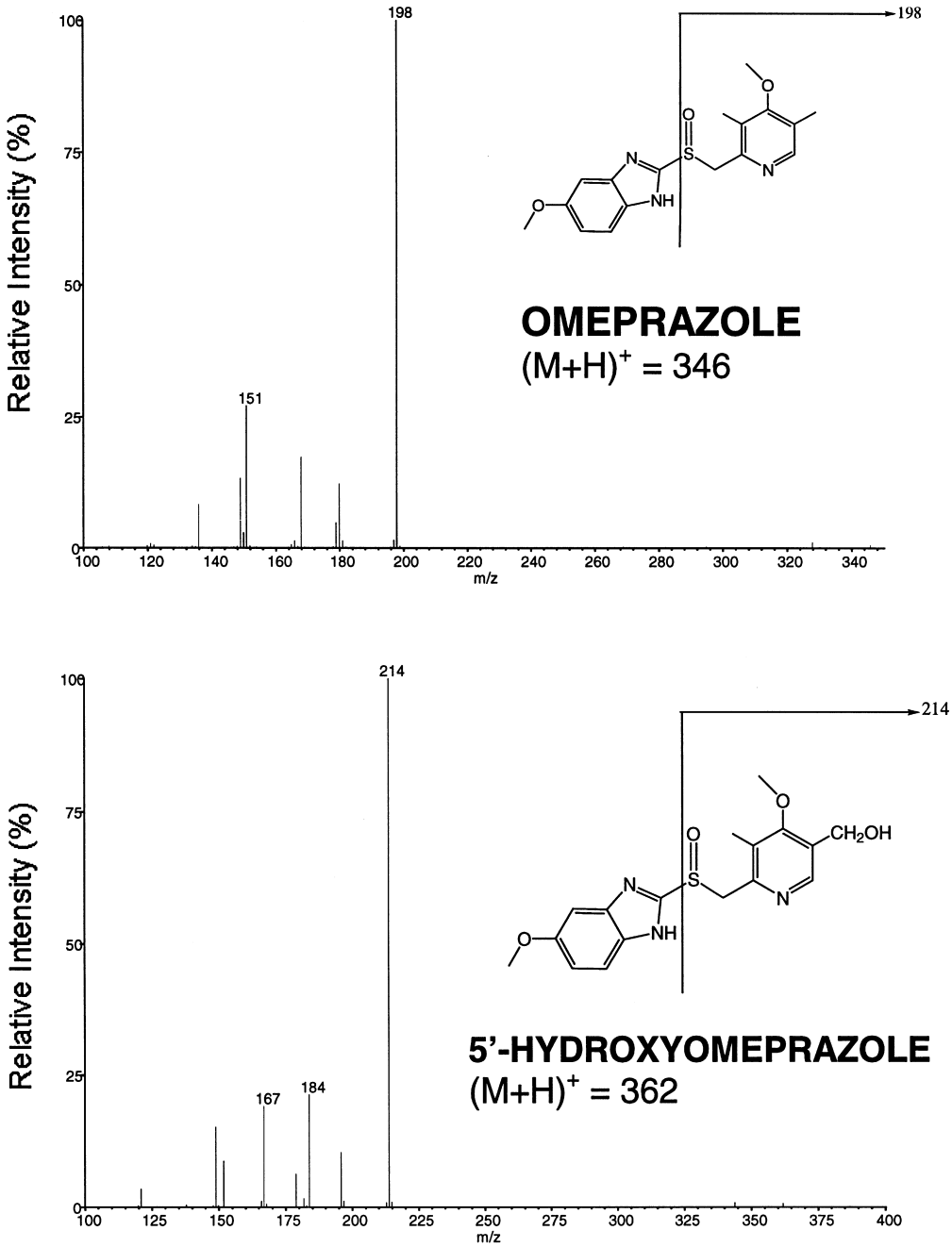

Fig. 5. Positive product ion mass spectra of the protonated molecular ions of omeprazole and 5′-hydroxyomeprazole, under the conditions used for the assay.

analyte recovery to about 80%. The lower recovery obtained with the vacuum manifold may be due to the fact that this procedure does not allow the elution solvent to remain in contact with the sorbent for a sufficient period of time in order to fully desorb the analytes.

Table 3
Concentrations of omeprazole and 5′-hydroxyomeprazole determined in plasma samples obtained 2 h after the oral administration of 20 mg omeprazole

Subject	Omeprazole concentration (ng/ml)	5′-Hydroxyomeprazole concentration (ng/ml)	Ratio of the concentration of omeprazole to that of 5′-hydroxyomeprazole
1	13.6	24.4	0.56
2	106.9	166.0	0.64
3	110.4	119.4	0.78
4	27.7	49.4	0.56
5	43.7	162.4	0.41
6	627.7	38.7	16.2

The SPE–LC–MS–MS procedure described here has been used to determine the concentrations of omeprazole and 5′-hydroxyomeprazole in plasma samples obtained 2 h after the oral administration of 20 mg of omeprazole (Table 3). Based on these results, subjects 1 to 5 would be classified as extensive metabolizers and would be expected to exhibit normal CYP 2C19 activity, while subject 6 would be classified as a poor metabolizer and would be expected to exhibit reduced CYP 2C19 metabolic activity. Thus, the assay, as described, appears to be suitable for determining the CYP 2C19 metabolic phenotype of subjects participating in clinical studies based on a measurement of their omeprazole/5′-hydroxyomeprazole plasma concentrations.

5. Conclusions

A method based on HPLC with tandem MS detection for the simultaneous determination of omeprazole and 5′-hydroxyomeprazole in human plasma has been developed. Exposure to toxic halogenated solvents during the sample preparation procedure has been eliminated through the utilization of SPE. The precision, accuracy, selectivity and sensitivity of the method makes it suitable for the analysis of samples collected during human clinical studies. The 11 min run-time of the assay is shorter than that of several published assays, thus allowing increased sample throughput. Additionally, the SPE step of the assay could potentially be automated in order to further improve sample throughput. Only 5% of the final extract was required to be injected into the LC–MS–MS system to achieve a limit of quantitation of 10 ng/ml for each analyte. Sensitivity of the assay could be further improved by reducing sample reconstitution volume and increasing injection volume. Such a sensitivity increase could be useful for studying the pharmacokinetics of lower doses of omeprazole. The assay has been successfully utilized to determine the CYP 2C19 metabolic phenotype of subjects participating in a clinical study.

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