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Analysis of omeprazole and 5-OH omeprazole in human plasma using hydrophilic interaction chromatography with tandem mass spectrometry (HILIC–MS/MS)—Eliminating evaporation and reconstitution steps in 96-well liquid/liquid extraction

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Abstract

Bioanalytical methods using liquid/liquid extraction (LLE) and liquid chromatography with electrospray tandem mass spectrometry (LC–MS/MS) are widely used. The organic extracts need to be evaporated and reconstituted, hampering further improvement of throughput and automation. In this study, we demonstrated a novel approach of eliminating these two steps in 96-well LLE by using hydrophilic interaction chromatography with MS/MS (HILIC–MS/MS) on silica column with high organic/low aqueous mobile phase. Omeprazole, its metabolite 5-OH omeprazole, and internal standard desoxyomeprazole, were extracted from 0.05 ml of human plasma using 0.5 ml of ethyl acetate in a 96-well plate. A portion (0.1 ml) of the ethyl acetate extract was diluted with 0.4 ml of acetonitrile and 10 μl was injected onto a Betasil silica column (50 mm × 3.0 mm, 5 μm) and detected by API 3000 and 4000 with (+) ESI. Mobile phase with linear gradient elution consists of acetonitrile, water, and formic acid (from 95:5:0.1 to 73.5:26.5:0.1 in 2 min). The flow rate was 1.5 ml/min with total run time of 2.75 min. The method was validated for a low limit of quantitation at 2.5 ng/ml for both analytes. The method was also validated for specificity, reproducibility, stability and recovery. Lack of adverse matrix effect and carry-over was also demonstrated. The inter-day precision and accuracy of the quality control samples at low, medium and high concentration levels were <4.4% relative standard deviation (R.S.D.) and 4.1% relative error (R.E.) for omeprazole, and 4.5% R.S.D. and 5.6% R.E. for 5-OH omeprazole, respectively.

Keywords: Omeprazole; 5-OH omeprazole; HILIC–MS/MS; Liquid/liquid extraction

1. Introduction

Omeprazole is often prescribed for treating gastroesophageal reflux disease. Its main active metabolite is 5-OH omeprazole. Numerous quantitative bioanalytical methods have appeared in literatures using HPLC with UV detection [1–11]. These methods, with large quantity of plasma samples used, have LLOQ at 5–10 ng/ml, which is slightly higher than what needed for our pharmacokinetic studies. The run time is usually quite long according to today's standard. Analysis of omeprazole and 5-OH omeprazole in biological fluids using liquid chromatography with electrospray tandem mass spectrometry (LC–MS/MS) was first reported by Woolf and Matuszewski [12]. The analytes were extracted from 1 ml of plasma by SPE using Waters Oasis cartridge and the analytical column was a Zorbax XDB C8 column with run time of 11 min. The LLOQ was 10 ng/ml for both analytes. The methanol eluate from SPE was evaporated and samples were reconstituted in mobile phase. Kanazawa et al. determined omeprazole and metabolites with 2.0 ml of plasma samples by LC–MS/MS using sonic spray ionization interface [13]. A LLOQ of 500 ng/ml is obtained using YMC-Pack Pro C18 column after a lengthy manual sample preparation and 25 min run time for analysis. Stenhoff et al. [14] reported a chiral LC–MS/MS analysis for omeprazole enantiomers in plasma using normal-phase chromatography on a Chiralpak AD column. The LLOQ is 10 nmol/l (equivalent

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to 3.45 ng/ml). 5-OH omeprazole was not measured in their study. A liquid/liquid extraction (LLE) was used to extract the analyte from plasma, using a 4 ml of 1% 2-butanol in dichloromethane–hexane (1:1, v/v). The organic extracts were evaporated to dryness and the residue was reconstituted with 1% acetonitrile in ethanol–hexane (3:7, v/v) which is compatible with the mobile phase. A LC–APCI-MS/MS method suitable for rapid pharmacokinetic screening was described by Colwell et al. [15]. A linear range of 1–1000 ng/ml using 0.100 ml of plasma sample with chromatographic run times of 2 min is obtained on a Spherisorb 50 mm × 4.6 mm CN column. They too employed a liquid/liquid extraction using ethyl acetate as the extraction solvent. The ethyl acetate extract was evaporated to dryness and residue was reconstituted with the mobile phase. More recently, LC–MS/MS of simultaneous detection of omeprazole and other anti-ulcer drugs in horse urine was reported by Chung et al. [16]. The assay requires 5 ml of urine samples and 20 min of cycle time for the analysis. A liquid/liquid extraction was also used to extract the analyte and once again a solvent evaporation and reconstitution was required.

All of above literature methods require steps of solvent evaporation and reconstitution. This process of evaporation and reconstitution is often the most lengthy and cumbersome of tasks [17]. Many analytes are also susceptible to loss due to thermal-degradation, adsorption, and evaporation. In HILIC on the silica column and with aqueous/organic mobile phase, the high organic eluent has a weaker elution strength than the mobile phase and thus the extract can be injected directly onto the column, eliminating the need for length evaporation and reconstitution steps, and increasing throughput [18].

In previous communications we reported feasibility of analysis of several polar analytes including omeprazole in plasma using direct injection of SPE eluent [19] or preliminary results of the LLE extraction [20]. We further expanded the work to include analysis of omeprazole and its metabolite 5-OH omeprazole and validated the method. Some of the technical challenges observed previously such as pulp-up of the 96-well mat when in contact with organic solvent vapor were effectively overcome in this current study. The current work is aimed to provide a novel approach for 96-well LLE without solvent evaporation and reconstitution, resulting in a significant saving of time.

2. Experimental

2.1. Chemicals and reagents

Omeprazole was purchased from USP (Rockville, MD, USA), 5-OH omeprazole was from SynFine Research (Richmond Hill, Ontario, Canada), and the internal standard desoxyomeprazole was synthesized at Covance. The chemical structures of these compounds are shown in Fig. 1. Acetonitrile, methanol, ammonium hydroxide (30%), ethyl acetate, acetic acid, all of HPLC grade, were from Fisher Scientific (St. Louis, MO, USA). Formic acid (96%) was from Sigma–Aldrich (St. Louis, MO, USA) and water, Type 1, was purified on a Barnstead system at Covance. Human plasma with sodium heparin as anticoagulant was from Biochemed (Winchester, VA, USA).

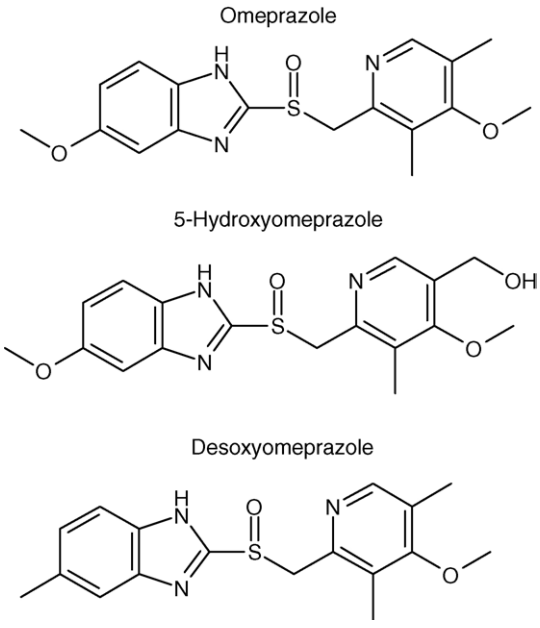

Fig. 1. Chemical structures of omeprazole, 5-OH omeprazole and internal standard (IS) desoxyomeprazole.

96-deep-well collection plates and dimpled sealing mats were from Axygen (Union City, CA, USA).

2.2. Preparation of standards and QC samples

The calibration standards (2.5, 5, 15, 75, 500, 1250, 2000 and 2500 ng/ml) and quality control samples (2.5, 7.5, 180, 1800 and 10,000 ng/ml) were prepared by adding appropriate amounts of stock or sub-stock solutions (in 1:1, methanol/water) into blank plasma. Aliquots of standards and QCs were transferred into pre-labeled 2 ml polypropylene vials with screw caps. Store all standards and QC samples in a freezer set to maintain −10 to −30 °C.

2.3. Sample preparation

Thaw samples, standards, and QCs at room temperature. Vortex-mix all samples for approximately 20 s. Aliquots of 0.05 ml of each sample, standard, and QC were transferred to 96-well Axygen deep-well collection plate using Packard Multiprobe II robotic liquid handler (Meriden, CT, USA). Internal standard solution (50 μl, 100 ng/ml in 1:1, methanol/water) was added to each sample except the blank sample. Ten microlitres of 2% ammonium hydroxide in water was added to each sample. Ethyl acetate (0.5 ml) was added to each sample and the plate was covered with a dimpled sealing mat. The plate was vortex-mixed for 10 min. The plate was then centrifuged at 3000 rpm and at 4 °C for 5 min. Using the Tomtec Quadra 96 workstation (Hamden, CT, USA), 0.1 ml of the ethyl acetate layer was transferred to an Axygen 96-well collection plate containing 0.4 ml of acetonitrile in each sample well. Mix 10 times by aspiration and dispersion on the Tomtec. The plate was then covered with a sealing mat and stored in a refrigerator at 2–8 °C.

2.4. HILIC–MS/MS method

The hydrophilic interaction chromatography with MS/MS (HILIC–MS/MS) system consisted of a Shimadzu 10ADVP HPLC system (Kyoto, Japan) and a PE Sciex API 3000 and 4000 tandem mass spectrometer (Concord, Ontario, Canada) with (+) ESI. The analytical column, Betasil silica of 5 μm, 50 mm × 3.0 mm i.d., was from Keystone Scientific (Bellefonte, PA, USA). The columns were maintained at 25 °C. The mobile phase was acetonitrile–water–formic acid (linear gradient elution, from 95:5:0.1 to 73.5:26.5:0.1 in 2 min). The flow rate was 1.0 ml/min for API 3000 and 1.5 ml/min for API 4000 without any eluent split.

The injection volume was 10 μl and run time was 2.75 min. The sample tray temperature was kept at 4 °C. The injection wash solution was methanol containing 1% acetic acid. The needle was rinsed prior to and after each injection with 0.50 ml of the wash solution. The multiple reaction monitoring (MRM) conditions for each analyte were optimized by infusing ca. 0.1 μg/ml of analyte in mobile phase. The Ionspray needle was maintained at 4.0 kV. The turbo gas temperature was 650 °C. Nebulizing gas, auxiliary gas, curtain gas, and collision gas flows were at instrument settings of 35, 35, 40 and 4, respectively. The declustering potentials (DP) for omeprazole, 5-OH omeprazole, and internal standard were at 40, 55 and 44, respectively. The mass spectrometer was operated under MRM mode with a collision energy (CE) of 17, 16 and 15 eV for omeprazole, 5-OH omeprazole and internal standard, respectively. The transitions (precursor → product) monitored were m/z 346 → 198 for omeprazole, 362 → 214 for 5-OH omeprazole and 330 → 198 for desoxyomeprazole. The dwell time was 200 ms for the analytes and 100 ms for internal standard. Both quadrupoles were maintained at unit resolution. Chromatograms were integrated using the Analyst version 1.4 software. A weighted 1/concentration^2 linear regression was used to generate calibration curves from standards and calculate the concentrations of quality control samples. At least 500 extracted samples were injected onto each column without any column regeneration. Peak shape and retention time remained unchanged.

2.5. Method validation

The full validation experiment followed "Guidance for Industry—Bioanalytical Method Validation" recommended by the Food and Drug Administration (FDA) of the United States (FDA, *Guidance for Industry*: *Bioanalytical Method Validation*, available at http://www.fda/cder/guidance/index.htm). Recovery was calculated by comparison of the analyte peak areas of extracted QC samples with those of post-extracted plasma blanks fortified with the known amount of analyte neat solutions. The method specificity was evaluated by screening six lots of blank plasma prior to the main validation batches. In this screening batch, six lots of plasma were fortified, individually, with the analytes at medium QC level, extracted and analyzed along with a calibration curve prepared in one of the six lots of plasma. The reproducibility of these six spiked samples is used to evaluate the presence or absence of interference, and the lot-to-lot variation. The sample injection sequence was randomly arranged through the entire curve except that the batch always started and ended with a calibration standard. A blank matrix sample was always injected after the highest standard to determine the injector carry-over.

3. Results and discussion

3.1. Method development for automated 96-well LLE and HILIC–MS/MS

Liquid/liquid extraction is an extraction technique for quantitative bioanalytical methods where analytes are extracted based on their partitioning between the aqueous and organic phases. Although recent advances in automated configuration have allowed use of LLE in 96-well format, the organic extracts inevitably need to be transferred to another 96-well plate for solvent evaporation and reconstitution [21–28]. Here, we report a novel approach of direct injection of organic extracts by using HILIC–MS/MS on silica columns with high organic/low aqueous mobile phases. Unlike normal phase LC where the water content in the mobile phase needs to be kept in minimal level, the presence of a significant amount of water (usually >5%) in the mobile phase is crucial for HILIC to maintain a stagnant enriched water layer on the surface of the stationary phase into which the analytes may selectively partition. HILIC–MS/MS was found to be an attractive alternative to reversed-phase columns and has been extensively used for quantitative bioanalytical analysis of polar compounds, as reviewed by Naidong [29].

Omeprazole, 5-OH omeprazole and the internal standard (desoxyomeprazole) were extracted from 0.05 ml of human sodium heparin plasma using LLE with 0.5 ml of ethyl acetate. The sample was made to basic pH by adding ammonium hydroxide. The extraction was performed in a deep-well 96-well Axygen plate by vortex-mixing. Various organic solvents were tested for their compatibility of being directly injected onto HILIC–MS/MS. MTBE, ethyl ether, and ethyl acetate were found to be compatible for direct injection. Typical organic solvents are usually too strong to be injected onto a reversed-phase column. However, these solvents are weak solvents for the silica column operating under the hydrophilic interaction chromatography with aqueous/organic mobile phase. These organic solvents are also miscible with acetonitrile and therefore can be diluted with acetonitrile and injected directly on column with no observable deterioration of peak shape. In order to inject at least 1–5 μl of sample solution, ethyl acetate extracts need to be diluted with acetonitrile (1:4, v:v). Typical injection volume is ∼10 μl. Although methyl butyl ether (MTBE) could be used to give cleaner extract, the drawback of using MTBE is that the 96-well mat available to us is not tight enough for preventing its eventual pulp-up caused by the vapor pressure of MTBE. And ethyl acetate was found to be significantly lower vapor pressure and pulp-up of the mat was not observed even after storing the plate in a refrigerator for 50 h.

High flow rate is applied to the LC separation. The possibility of running high flow rate on the silica column is due to its very low back-pressure. The mobile phase for omeprazole and

its metabolite is water–acetonitrile–formic acid with a linear gradient elution. We again have successfully demonstrated the reproducibility of running gradient elution on the silica column [30].

3.2. Validation

Six lots of blank plasma were tested for matrix interference. The chromatographic regions in which the analyte and inter-

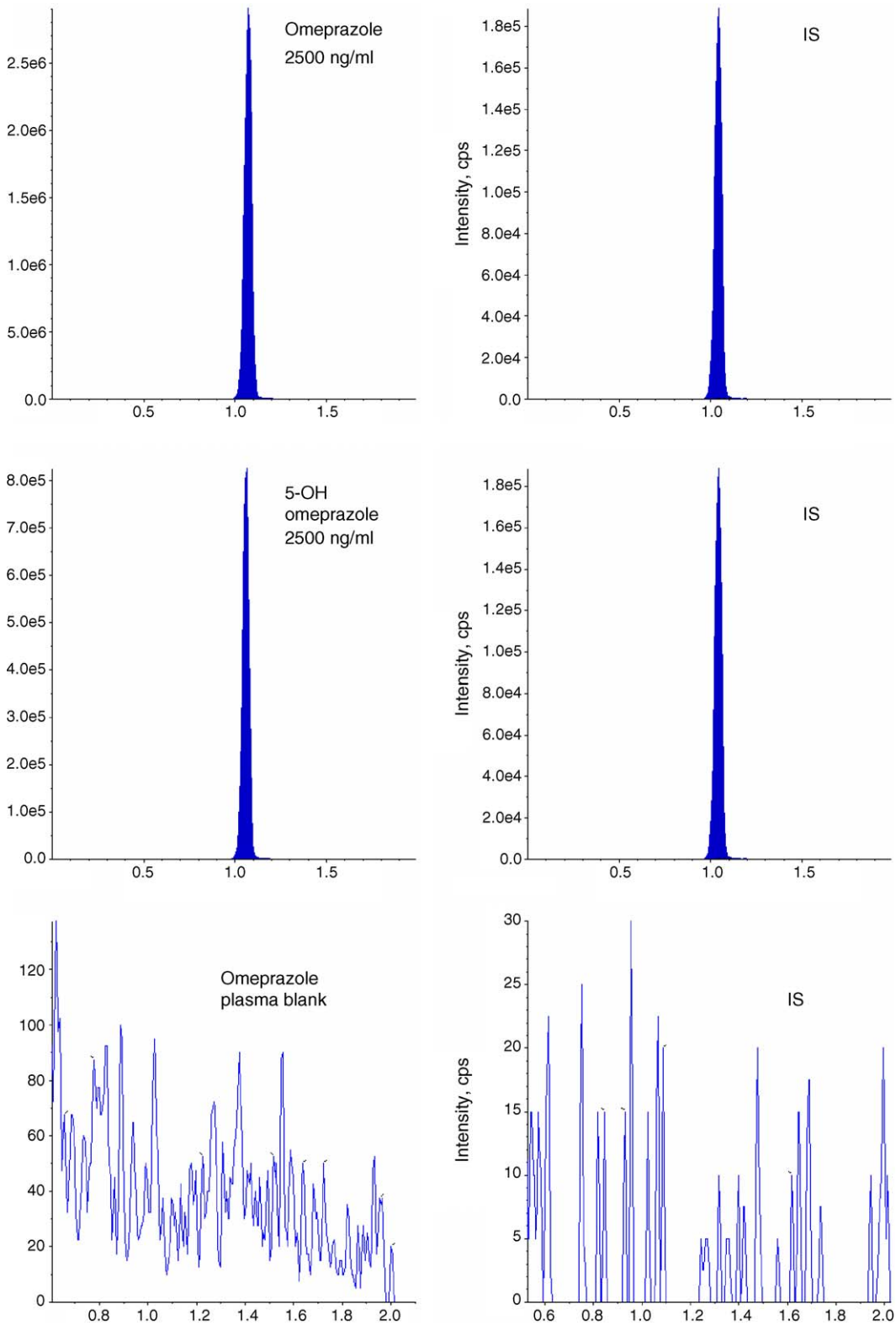

Fig. 2. Chromatogram of three consecutive injections of (A) extracted calibration standard at 2500 ng/ml of omeprazole and 5-OH omeprazole, (B) extracted blank plasma sample, and (C) extracted low limit of quantitation (LLOQ) at 2.50 ng/ml of omeprazole and 5-OH omeprazole. IS: internal standard (desoxyomeprazole).

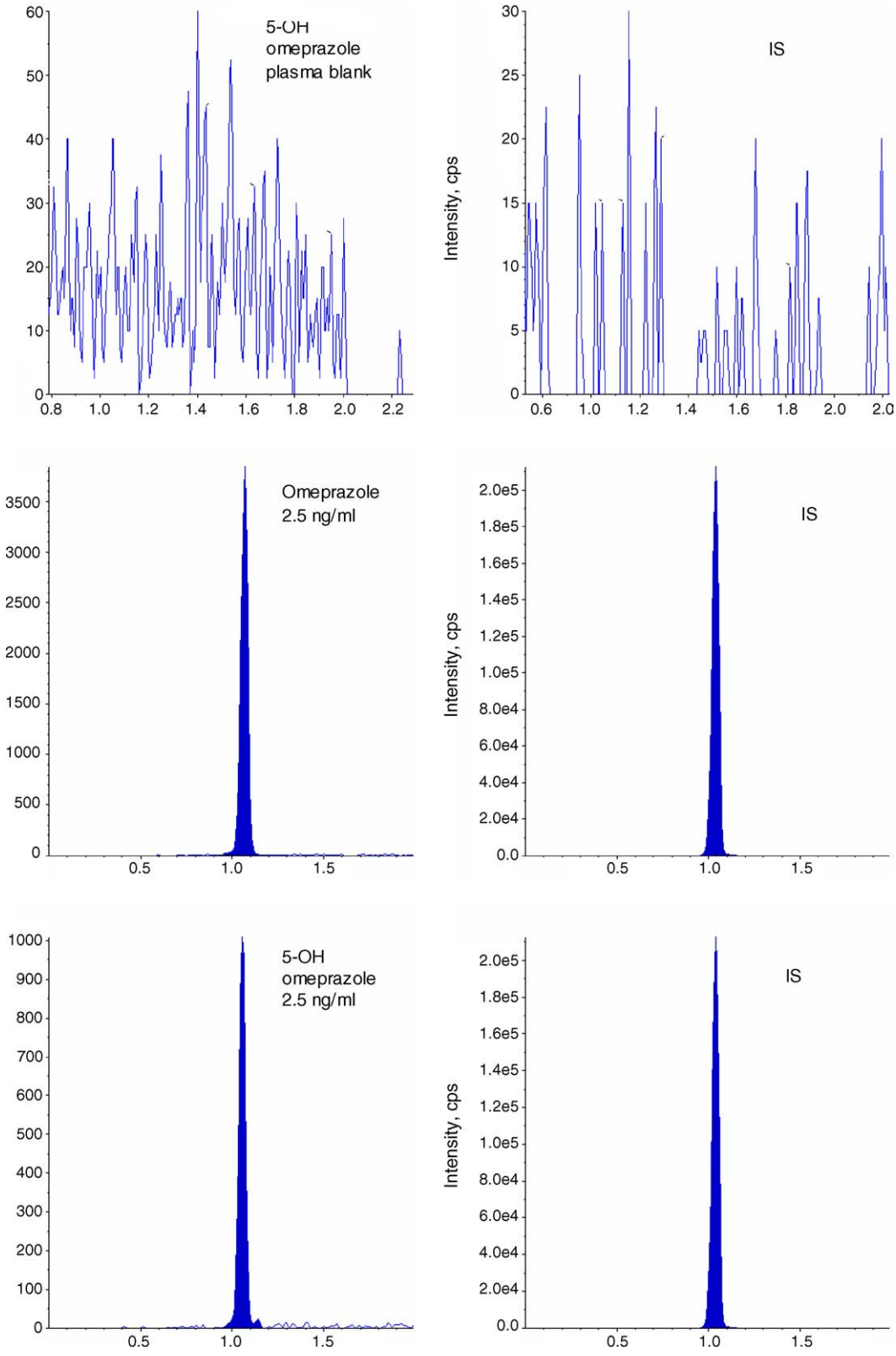

Fig. 2. (*Continued*).

nal standard peaks are observed were free from interference. Fig. 2 shows the chromatograms of three consecutive injections of extracted samples (extracted calibration standard at 2500 ng/ml, extracted blank, and extracted low limit of quantitation at 2.50 ng/ml) on a silica column. Minimal carryover from autosampler was observed occasionally. When observed, carry over was less than 5% of lower quantitation limit. Desoxyomeprazole is synthesized from omeprazole, potential interfer-

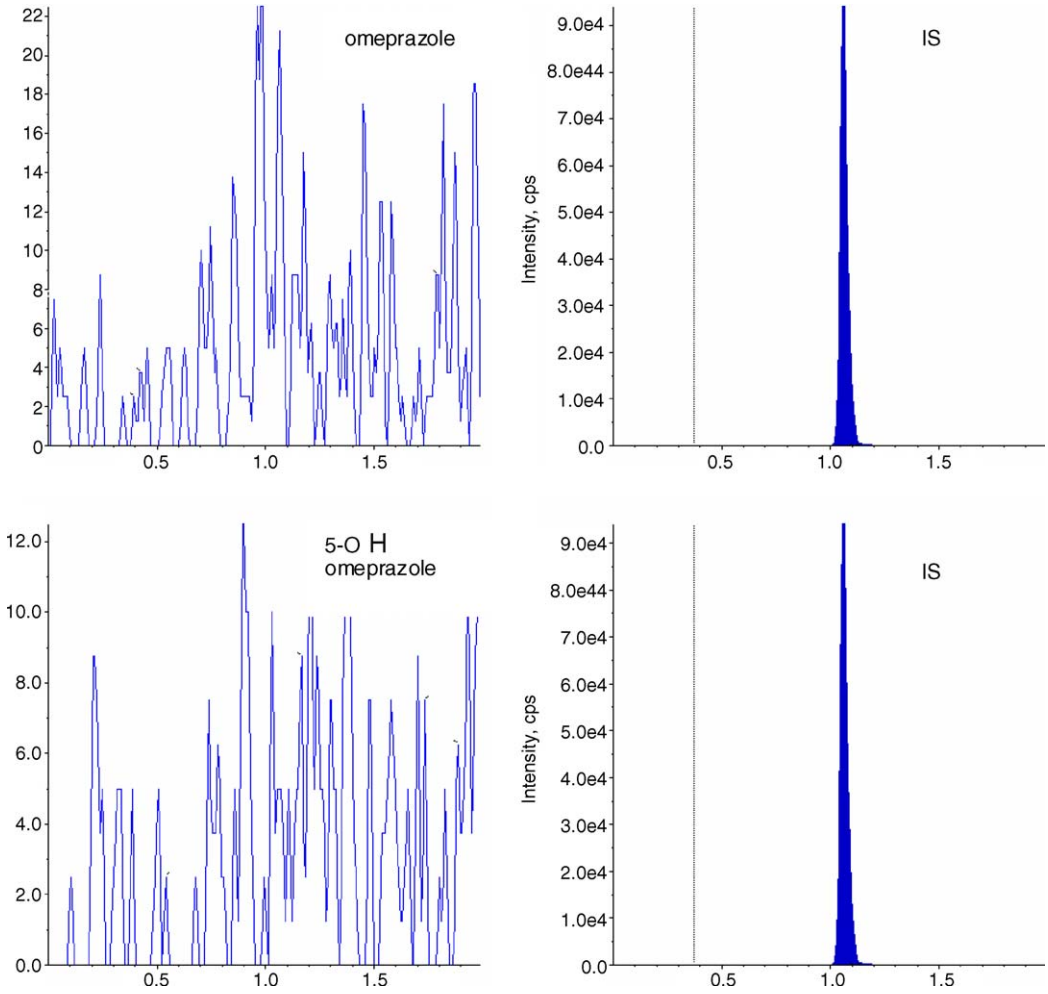

Fig. 3. Chromatogram of extracted blank plasma spiked with desoxyomeprazole only.

ence in the analyte signal that could be caused by the internal standard was verified. Fig. 3 shows a chromatogram of blank plasma spiked with only desoxyomeprazole. This chromatogram demonstrates that there is no detectable residual omeprazole in the internal standard at the concentration levels used. When the samples were spiked with omeprazole and 5-OH omeprazole at 100 ng/ml, the relative standard deviation (R.S.D.) were 1.0 and 1.5%, respectively. These tight R.S.D. values indicate no significant lot-to-lot variation in matrix effects. Consistent peak areas for both omeprazole and 5-OH omeprazole were obtained for all six lots of plasma. A post-column infusion experiment, as described by King and coworkers [31], was used to investigate the matrix effect. Omeprazole and 5-OH omeprazole were constantly post-column infused into the mobile phase while an extracted blank matrix was injected and chromatographed. If there were any signal suppression, a negative signal from the baseline would be observed. With ethyl acetate as the extraction solvent, no negative signal from all six individual blank matrices was observed. Matrix suppression was therefore not observed. In this screening batch, six lots of plasma were fortified, individually, with the analytes at medium QC level, extracted and analyzed along with a calibration curve prepared in one of the six lots of plasma. The reproducibility of these six spiked samples is used to evaluate the presence or absence of interference, and the lot-to-lot variation. Clarithromycin, a co-administered medicine, is also tested for the interference. Clarithromycin was spiked into blank matrix and QC samples at the concentration of C_{max} (2500 ng/ml). No interference peak was observed at the region of analytes of interest in blank extracts. Spiked QC samples in triplicates were analyzed along with a calibration curve. The spiked QC samples yield similar precision and accuracy as the nonspiked QC samples indicated that the presence of clarithromycin showed no adverse effect on the quantitation of omeprazole and 5-OH omeprazole.

Recovery was determined by comparing the MS response of the extracted sample with those spiked (post-extraction) into a blank matrix. Because both samples have the matrix ingredients present, the matrix effects can be considered the same for extracted samples and post-extraction spiked samples. Any differences in responses can be considered caused by extraction recovery. Recovery for omeprazole is 94% at 7.5 ng/ml (R.S.D.% = 3.1, n = 6), 87% at 180 ng/ml (R.S.D.% = 3.7, n = 6), and 98% at 1800 ng/ml (R.S.D.% = 3.0, n = 6). Recovery for 5-OH omeprazole is 60% at 7.5 ng/ml (R.S.D.% = 3.4, n = 6), 54% at 180 ng/ml (R.S.D.% = 4.6, n = 6), and 63% (R.S.D.% = 2.8,

Table 1
Precision and accuracy of quality control samples

	Intra-day (n = 6)					Inter-day (n = 18)		
	2.50 ng/ml	7.50 ng/ml	180 ng/ml	1800 ng/ml	10000[a] ng/ml	7.50 ng/ml	180 ng/ml	1800 ng/ml
Omeprazole								
Mean	2.36	7.64	177	1820	10100	7.81	179	1870
R.S.D. (%)	4.0	3.9	1.6	1.8	2.7	4.4	−0.6	+3.9
R.E. (%)	−5.6	+1.9	−1.7	+1.1	+1.0	+4.1	−0.6	+3.9
5-OH omeprazole								
Mean	2.73	7.81	179	1830	10100	7.92	177	1830
R.S.D. (%)	8.1	4.4	0.9	3.3	2.7	4.4	4.5	2.4
R.E. (%)	9.2	+4.1	−0.6	+1.7	+1.0	+5.6	−1.7	+1.7

[a] Samples were diluted 10-fold with blank plasma prior to analysis.

n = 6). The overall mean recovery for the internal standard is 92%.

For all three validation curves, the results for all calibration standards showed a <7.3% R.S.D. and <9.3% R.E. for omeprazole, and <12.7% R.S.D. and <8.7% R.E. for 5-OH omeprazole. The linear correlation coefficient was >0.9976 for omeprazole and >0.9965 for 5-OH omeprazole over the curve range of 2.50–2500 ng/ml. The precision and accuracy data for QC samples are summarized in Table 1. The data show that this method is consistent and reliable with low R.S.D. and R.E. values. For the LLOQ QCs (2.50 ng/ml), the R.S.D. (n = 6) of the measured concentration was 4.0% for omeprazole and 8.1% for 5-OH omeprazole. The relative errors of the mean of the measured concentrations were −5.6% for omeprazole and +9.2% for 5-OH omeprazole. The S/N ratio at the LLOQ (2.5 ng/ml) level is 880 using only 0.050 ml of plasma sample and after five-fold dilution of its LLE extracts.

The stability tests were designed to cover the anticipated conditions that the clinical samples may experience. Stabilities of sample processing (freeze/thaw, bench-top and storage), and chromatography (extracts) were tested and established. The results are summarized in Table 2. Four freeze/thaw cycles and ambient temperature storage of the QC samples for up to 24 h prior to analysis, appeared to have little effect on the quantitation. QC samples stored in freezers at −20 °C remained stable for at least 39 days. Extracted calibration standards and QC samples were allowed to stand at 2–8 °C for 52 h prior to injection. No effect on quantitation of the calibration standards or QC samples was observed. Stock solutions of omeprazole and 5-OH omeprazole were stable for at least 6 h at room temperature and for at least 43 days at 2–8 °C.

Table 2
Stability of the samples (n = 6)

	Concentration (ng/ml)					
	Omeprazole			5-OH omeprazole		
	7.50	180	1800	7.50	180	1800
Four freeze/thaw cycles						
Mean	7.74	180	1840	7.78	184	1820
R.S.D. (%)	1.7	1.6	1.0	4.9	1.5	2.1
R.E. (%)	+3.2	0.0	+2.2	+3.7	+2.2	+1.1
24 h bench-top						
Mean	7.88	178	1840	8.10	181	1850
R.S.D. (%)	1.4	1.6	1.9	3.1	2.0	1.4
R.E. (%)	+5.1	−1.1	+2.2	+8.0	+0.6	+2.8
−20 °C for 39 days						
Mean	7.66	170	1790	8.17	175	1820
R.S.D. (%)	5.1	3.8	2.4	3.8	3.3	4.0
R.E. (%)	+2.1	−5.6	−0.6	+8.9	−2.8	+1.1
52 h extract						
Mean	7.56	172	1830	7.89	173	1790
R.S.D. (%)	3.6	5.2	2.9	4.6	4.4	2.8
R.E. (%)	0.8	−4.4	+1.7	+5.2	−3.9	−0.6

4. Conclusion

A high-throughput HILIC–MS/MS method for the measurement of omeprazole and 5-OH omeprazole in human plasma had been successfully developed and has demonstrated the novel approach of direct injection of organic extracts by using hydrophilic interaction chromatography with MS/MS on silica columns with high organic/low aqueous mobile phases. The ethyl acetate extracts is injected directly or after dilution with acetonitrile if necessary, and as a result the extraction throughput was significantly increased. A batch of 96 samples was processed in less than 1 h in comparison with at least 4 h needed using manual LLE in glass tubes. In addition to the time saving due to elimination of the manual evaporation and reconstitution steps, thus facilitating better automation and higher throughput, the advantages of this novel approach include avoiding loss of compound during evaporation and reconstitution due to instability, evaporation, or adsorption. The very low column back-pressure generated under HILIC conditions also allowed higher mobile phase flow rate to further improve throughput. The sensitivity of the method was significantly higher than what can be achieved on a typical reversed-phase LC–MS/MS due to the low aqueous and high organic nature of the mobile phase used for HILIC–MS/MS. Reported here is the simultaneous determination of omeprazole and 5-OH omeprazole using hydrophilic interaction chromatography with MS/MS. The method described is a simple, fast, very sensitive and automated LLE extraction with direct injection of 10 μl or less of 96-well organic extracts. Analytes are detected at the LLOQ level of

2.5 ng/ml after five-fold dilution using only 0.05 ml of plasma sample. It has been successfully used for clinical sample analysis. The validated approach of liquid/liquid extraction and the direct injection onto HILIC–MS/MS can be expanded to the analysis of many other polar analytes.

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