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Rabeprazole analysis in human plasma by fully validated HPLC assay

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ABSTRACT

A simple, rapid, and precise high performance liquid chromatography (HPLC) assay for rabeprazole measurement in human plasma was developed, validated, and used to study the stability of rabeprazole under various laboratory conditions. After extraction with the *tert*. butyl methyl ether, rabeprazole and lansoprazole (internal standard, IS) were separated at 4.8 and 8.7 minutes, respectively, on a Nova-Pak C_{18} cartridge at room temperature (RT), and detected by a photodiode array detector set at 284nm. The mobile phase consisted of 0.05% dibasic sodium phosphate, acetonitrile, and triethylamine (65:35:0.005, v: v: v) and the run time was 10 min. The response was linear over the range of 0.01-1.60 µg/ml. Extraction recovery and intra- and inter-run bias were ≥ 90% (mean 92%), ≤9% and <10%, respectively. Rabeprazole was stable in plasma for 24 h at RT (≥ 92%), 10 weeks at -20°C (≥ 95%), and after 3 cycles of freeze at -20°C and thaw at RT (100%). In extracted samples, rabeprazole was stable for 24 h at RT (≥ 93%) and 48 h at -20°C (92%). Rabeprazole stock solution (1 mg/ml in methanol) was stable for 48 h at RT (100%) and 10 weeks at -20°C (98%). Rabeprazole level was 0.26µg/ml five hours after the ingestion of a single 20mg dose. The data indicate that the described assay is suitable for therapeutic drug monitoring and bioequivalence studies in humans.

KEYWORDS

Rabeprazole;
Lansoprazole;
HPLC;
Stability;
Recovery;
Validation.

INTRODUCTION

Rabeprazole sodium, 1H-Benzimidazole, 2-[[[4-(3-methoxypropoxy)-3-methyl-2-pyridinyl]- methyl] sulfinyl]-monosodium salt (CAS number: 117976-89-3), belongs to the proton pump inhibitor group[1], proton pump inhibitors inhibit acid secretions and produce sustained elevations in gastric pH, making them the treatment of choice for acid peptic disease[2]. Rabeprazole oral bioavailability is about 52%, it circulates 95% to 98% bound to plasma proteins, and has 5 inactive metabolites[3]. A peak plasma concentration around 0.48µg/ml is achieved after oral administration of a single usual therapeutic dose of 20mg[1]. Peak plasma concentrations and extent of bioavailability are linear over an oral dose range of 10 to 40mg[1]. Rabeprazole is rapidly degraded in acidic medium and is more stable in alkaline solutions[4].

A literature review revealed few analytical methods for the determination of rabeprazole in biological fluids, including high performance liquid chromatography (HPLC)[5-11] and liquid chromatography coupled with mass spectrophotometry[12,13]. These methods suffered from low recovery[6,8,12], complicated sample prepara-

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tion[7], or long chromatographic run time[5,6,10]; were not validated using human plasma[5,10]; lacked sensitivity[5,8,10]; required column switching[6,9,10]; or solid phase extraction[8,11]; or did not address rabeprazole stability[5,7,10].

The aims of the study were to 1) establish a simple, fully validated, rabeprazole HPLC assay in human plasma with a quantitation limit suitable for bioequivalence studies and therapeutic level monitoring, and 2) determine the stability of rabeprazole under various clinical laboratory conditions.

EXPERIMENTAL

Apparatus

The liquid chromatograph consisted of Waters Alliance 2690 Separations Module; an 8×100mm, 4μm (particle-size) Nova-Pak C_{18} cartridge column; a Guard Pak pre-column module with Nova-Pak C18, 4μm insert; and Waters 996 photodiode array detector (Water Associates, Milford, MA, USA) set at 284nm. Data were collected with a Pentium III computer using Millennium^{32} Chromatography Manager Software (Water Associates, Milford, MA, USA).

Chemicals and reagents

Rabeprazole sodium standard (Figure 1a) was supplied by Jamjoom Pharma, Jeddah, Saudi Arabia, and the internal standard (IS) lansoprazole (CAS number; 103577-45-3) (Figure 1b) was purchased from Sigma-Aldrich, Louis, MO, USA. Dibasic sodium phosphate was purchased from Sigma Chemical Company, St. Louis, MO, USA. *Tert.* butyl methyl ether was purchased from FlukaChemie AG, Switzerland. Acetonitrile, methanol, triethylamine, diethaylamine, and phsphoric acid (all HPLC grade) were purchased from Fisher Chemical Scientific, Fair Lawn, New Jersey, USA. Water for HPLC was prepared by reverse osmosis and further purified by passing through a Milli-Q System obtained from Millipore Co. (Bedford, MA, USA).

Chromatographic conditions

The mobile phase consisted of 0.05% dibasic sodium phosphate (pH adjusted to 7.0 with phosphoric acid), acetonitrile, and triethylamine (65:35:0.005, v:v:v).

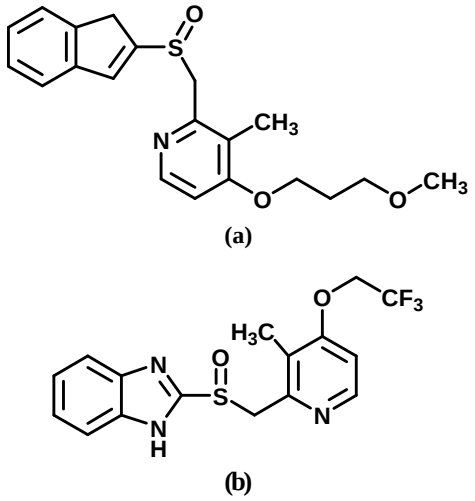

Figure 1 : Chemical structures of rabeprazole (a) and the internal standard, lansoprazole (b)

It was filtered through a 0.22 μm size membrane filter (Millipore Co., Bedford, MA, USA), degassed, and delivered at a flow rate of 2.0ml/min. The autosampler was programmed to inject 100 μl into the chromatograph with a run time of 10 min.

Preparation of stock and working solutions

Rabeprazole (1mg/ml) stock solution was prepared in 0.1% diethylamine in methanol and used for stability studies and to prepare a 2.0 μg/ml working solution in plasma. The working solution was prepared weekly to construct calibration curve and quality control (QC) samples. Lansoprazole working solution (10μg/ml) was prepared weekly in mobile phase from a stock solution in methanol (1mg/ml).

Calibration standard/ quality control samples

Calibration standards were prepared by mixing appropriate volumes of rabeprazole working solutions with blank human plasma to produce final concentrations of blank, zero (blank plasma spiked with IS only), 0.01, 0.02, 0.04, 0.08, 0.10, 0.32, 0.64, 1.28, and 1.60μg/ml. QC samples were prepared by mixing appropriate volumes of rabeprazole working solution in blank human plasma to produce final concentrations of 0.01, 0.03, 0.80, and 1.44μg/ml. Samples were vortexed for 20 seconds, and aliquots of 1 ml of calibration standards and QC samples were transferred into 13 x 100 mm Teflon-lined, screw-capped, borosilicate glass, culture tubes (Fisher Scientific Co., Fairlawn, NJ, USA), and stored at -20°C.

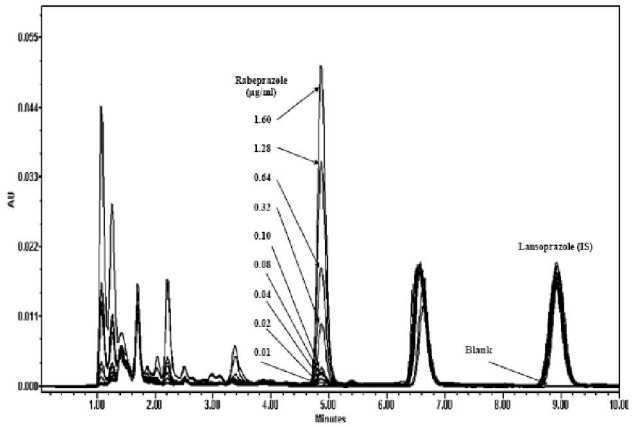

Figure 2 : Overlay of rabeprazole calibration curve chromatograms spiked with 0.00 (B), 0.01, 0.02, 0.04, 0.08, 0.10, 0.32, 0.64, 1.28, or 1.60µg/ml of rabeprazole and 0.70µg/ml of lansoprazole (IS)

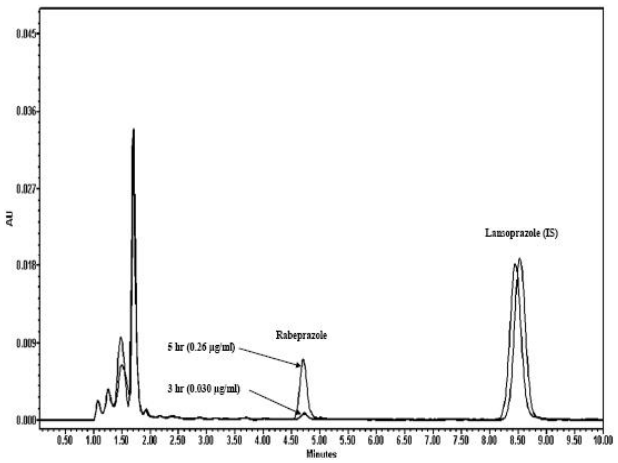

Figure 3 : Overlay of two chromatograms of volunteer plasma samples 3 and 5 hours after oral administration of a single 20mg rabeprazole tablet

Sample preparation

Aliquots of 1ml of calibration standard or QC samples in 13×100mm Teflon-lined, screw-capped, borosilicate glass, culture tubes were allowed to equilibrate to room temperature. To each tube, 70 µl of the 10µg/ml IS working solution was added and vortexed for 10 seconds. After the addition of 5.0ml of *tert.* butyl methyl ether, the mixture was vortexed again for 5 min and then centrifuged for 15 min at 13200 rpm at room temperature. The organic layer was carefully collected and dried under a gentle stream of nitrogen at 40°C; and the residue was reconstituted in 250µl mobile phase and centrifuged at 3500 rpm for 5 min. The supernatant layer was carefully transferred into autosampler vials and 100µl were injected.

Stability studies

Stability of rabeprazole in plasma: Adequate number of aliquots of two QC samples (0.03, and 1.44µg/ml) was prepared. Aliquots were analyzed in 5 replicates immediately (baseline), after being processed and stored at room temperature for 24 h or at -20°C for 48 h (auto-sampler stability), after being allowed to stand on the bench-top for 8 or 24 h at room temperature before processing (counter stability), after being stored at -20°C for 10 weeks before processing (long term freezer stability), or after being stored at -20°C for 24 h and then left to completely thaw unassisted at room temperature before processing (with the cycle repeated three times, freeze-thaw stability).

Stock solutions stability

Five aliquots of the stock solutions of rabeprazole and the IS were analyzed (after dilution to 10µg/ml in mobile phase) at baseline, after storage for 48 h at room temperature, or after storage at -20°C for 10 weeks. Stability of the working solutions of rabeprazole and the IS, were evaluated up to 2 weeks at -20°C.

Assay validation method

The procedures used for validation are as described in the US Food and Drug Administration (FDA) bioanalytical method validation guidance[14].

RESULTS

Optimization of chromatographic conditions

During analytical method development, different combinations of the components of the mobile phase, different pH, and different flow rates were investigated to optimize separation of rabeprazole and the IS. A mobile phase composed of 0.05% dibasic sodium phosphate, acetonitrile, and triethylamine (65:35:0.005, v: v: v) was found best to achieve adequate separation, minimize background absorbance, and avoid peak tailing. In order to improve specificity and detection limit, and minimize interference from plasma or solvent system, we chose the best absorbance wavelength based on photodiode array extracted spectra (Figure 2) and preformed the analysis at 284nm. Under the described conditions, rabeprazole and the IS were resolved within

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TABLE 1: Specificity of rabeprazole assay

Drug Name	Retention Time (minutes)
Rabeprazole	4.8
Lansoprazole	8.7
Diclofenac sodium	2.5
Aspirin	1.8
Acetaminophen	1.3
Ranitidine	1.9
Nicotinic acid*	1.2
Ascorbic acid*	1.3
Caffeine*	1.8

1mg/ml solutions in methanol or water* were diluted in the mobile phase to 10μg/ml and 100μl were injected

a run time of 10 minutes, with a retention time of 4.8 and 8.7 minutes, respectively.

Linearity

Linearity was determined in the range of 0.01-1.60μg/ml using ten calibration curves. The data were analyzed by linear regression using the formula: Conc. = a + b (PHR), where Conc. is the concentration of rabeprazole, a is the intercept, b is the slope, and PHR is the peak height of rabeprazole divided by the peak height of the IS. The concentrations of the calibration standards of the ten calibration curves were back-calculated using the individual regression lines. Linearity studies (n = 10) showed a mean (SD) for R^2 of 0.9992 (0.0011), a slope of 0.6569 (0.0471), and an intercept of 0.0005 (0.0027) for the range of 0.01 to 1.60μg/ml. Figure 2 depicts an overlay of chromatograms of a representative standard curve. Figure 3 shows an overlay of two chromatograms of plasma samples collected from a healthy volunteer 3 and 5 h after the oral administration of a single 20mg rabeprazole tablet. Reported peak rabeprazole level after the oral administration of a therapeutic dosage of 20mg once daily for 7 days, was 0.48μg/ml after the first dose[1] and was 0.41μg/ml after the last dose[9], indicating that the described method is suitable for therapeutic drug monitoring.

Limit of detection

The limit of detection (defined as three times the baseline noise) for rapebrazole, was 0.005μg/ml.

Specificity

To evaluate specificity, we screened seven frequently used medications (10μg/ml in mobile phase) and six different batches of human plasma. All batches of blank plasma were free from interfering components. None of the seven medications co-eluted with rabeprazole or the IS (TABLE 1).

TABLE 2 : Extraction recovery of Rabeprazole and Lansoprazole

Nominal Concentration (μg/ml)	Plasma		Mobile phase		**
	*Mean peak height	SD	*Mean peak height	SD	Recovery (%)
Rabeprazole					
0.01	327	17	363	8.8	90
0.03	814	9.1	894	7.5	91
0.80	19328	449	20959	82	92
1.44	31555	482	32928	21	96
Lansoprazol					
0.70	15944	438	17699	95	90

***Mean peak height of 5 replicates. ** Mean peak height of spiked plasma samples divided by mean peak height of spiked mobile phase samples x 100. SD, standard deviation**

Recovery

The extraction recovery of rabeprazole was determined by dividing mean peak areas of five replicates of four QC samples (0.01, 0.03, 0.80, and 1.44μg/ml) prepared in plasma (as described under sample preparation), by mean peak areas of five replicates of equivalent concentrations prepared in the mobile phase. The recovery of the IS was determined similarly at a concentration of 0.70μg/ml. The results of the extraction recovery studies of rabeprazole and the IS are presented in TABLE 2. Recovery was ≥ 90% (mean 92%) for rabeprazole and 90% for the IS.

Precision and bias

Precision was calculated as coefficient of variation (standard deviation divided by mean measured concentration×100), and bias as the absolute value of (1 minus mean measured concentration divided by nominal concentration)×100. The intra-run and inter-run precision and bias of rabeprazole were determined by analyzing four QC samples: 0.01, 0.03, 0.80, and 1.44μg/ml over three days (TABLE 3). Intra-run precision and bias (n = 10) ranged from 4 % to 5.6 % and from 3 % to 9 %, respectively. The inter-run precision and bias (n = 20) ranged from 3.7% to 5.8% and from

TABLE 3 : Intra-run and inter-run accuracy and precision of rabeprazole assay

Nominal Concentration (µg/ml)	Intra-run (n=10)				Inter-run (n=20)			
	Mean measured concentration (µg/ml)	SD	Precision (CV*, %)	**Bias (%)	Mean measured concentration (µg/ml)	SD	Precision (CV*, %)	**Bias (%)
0.01	0.0109	0.0006	4.9	9	0.0110	0.0006	5.8	10
0.03	0.0316	0.0013	4.0	5	0.0320	0.0012	3.7	7
0.80	0.7741	0.0430	5.6	3	0.7787	0.0349	4.5	3
1.44	1.3752	0.0661	4.8	4	1.3883	0.0593	4.3	4

*Coefficient of variation (CV) = standard deviation (SD) divided by mean measured concentration100. **Bias = 1 minus mean measured concentration divided by nominal concentration×100

TABLE 4 : Stability of rabeprazole in plasma samples and stock solution

	*Plasma samples								**Stock solution	
	Unextracted			Extracted		Freeze-thaw				
Nominal concentration (µg/ml)	8 h RT	24h RT	10 wks –20^{0}C	24h RT	48 h –20^{0}C	One cycle	Two cycles	Three cycles	48 h RT	10 wks –20^{0}C
0.03	94	92	95	100	100	97	100	100		
1.44	100	95	98	93	92	100	100	100	100	98

Stability (%) = Mean measured concentration (n = 5) at the indicated time divided by mean measured concentration (n = 5) at baseline x 100. *Spiked plasma samples analyzed immediately (baseline, data not shown), after 8 or 24 hours at room temperature (8 h RT and 24 h RT), or 10 weeks at -20°C (10 wks -20°C); analyzed after storage of the extract for 24 hours at room temperature (24 h RT) or 48 hours at -20°C (48 h -20°C); or analyzed after 1 to 3 cycles of freezing plasma at -20°C and thawing at room temperature (freeze-thaw).** Rabeprazole 1 mg/ml in methanol

3 % to 10 %, respectively.

Stability

The results of rapebrazole stability studies under common laboratory storage conditions of plasma and extracted samples was investigated (TABLE 4). The data indicate that: 1) rapebrazole in plasma was stable for at least 24 h at room temperature and 10 weeks at -20°C, 2) in extracted samples, rapebrazole was stable for at least 24 h at room temperature and 48 h at -20°C, 3) rapebrazole in plasma was stable after at least three cycles of freeze at -20°C and thaw at room temperature, and 4) rapebrazole in methanol (1mg/ml) was stable for at least 48 h at room temperature or 10 weeks at -20°C. The IS in methanol (1mg/ml) was also stable for at least 48 h at room temperature or 10 weeks at -20°C (94% and 96%, respectively). Further, the working solutions of rabeprazole and the IS (2µg/ml in plasma and 10µg/ml in mobile phase, respectively) were stable for at least 2 weeks at -20°C (101% and 99%, respectively).

Robustness

The robustness of the proposed method was evaluated by slightly altering the strength of phosphoric acid and amount of acetonitrile in mobile phase. No significant effects were observed. Further, the chromatographic resolution and peak responses were stable over about 1200 injections of extracted plasma samples using one column.

DISCUSSION

Few assays of rabeprazole in plasma have been previously reported[5-11]. Takakuwa et al.[5] and Mano et al.[10] described a gradient enantioselective HPLC method for determination of rabeprazole in dog plasma with a chromatographic run time of 37 min and 25 min, respectively, and a limit of quantification of 30µg/ml. Tsukasa et al.[6] described an HPLC assay in human plasma that required 20 min chromatographic run time and column switching with a recovery of rabeprazole of 78%. Nakai et al.[7] determined rabeprazole and its metabolites in human plasma but did not examine rabeprazole stability during the analytical process. Further, sample preparation was complex, involving double extraction. Ramakrishna et al.[8] described an HPLC assay in human plasma that requires a long sample processing time and costly solid phase extraction cartridges

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with a lower limit of quantification of 20µg/mL and a recovery of 85%. The HPLC assay in human plasma described by Sonu et al.[9] involved minimal sample preparation, however, it required column switching technique. The HPLC mass spectrophotometry assay of Jinchang et al.[12] had an average recovery of 84 % rabeprazole from human plasma.

We describe a rapid, simple, accurate, and precise HPLC assay for the determination of therapeutic levels of rabeprazole in human plasma. The assay has the following advantages: simple sample preparation consisting of one-step liquid-liquid extraction, simple equipment, short run time, high sensitivity, and high extraction recovery. Using the assay, we obtained extensive data on stability of rabeprazole under various laboratory conditions, including three freeze thaw cycles, 10 weeks storage at -20°C in plasma or methanol, as well as stability of rabeprazole in extracted samples and stability of rabeprazole and the internal standard working solutions. Finally, we used the assay to determine rapebrazole level in a volunteer sample[1]. Although, the assay doesn't measure rabeprazole metabolites and is not an enantioselective, rabeprazole metabolites are inactive and measuring enantiomers is not clinically relevant. The data indicate that the described assay is suitable for therapeutic drug monitoring and bioequivalence studies in humans.

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