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# Determination of esomeprazole and its two main metabolites in human, rat and dog plasma by liquid chromatography with tandem mass spectrometry

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**Abstract**

A LC-MS/MS method was developed for quantitative determination of esomeprazole, and its two main metabolites 5-hydroxyesomeprazole and omeprazole sulphone in 25 μL human, rat or dog plasma. The analytes and their internal standards were extracted from plasma into methyl *tert*-butyl ether - dichloromethane (3:2, v/v). After evaporation and reconstitution of the organic extract the analytes were separated on a reversed-phase LC column and measured by atmospheric-pressure positive ionisation MS. The linearity range was 20–20,000 nmol/L for esomeprazole and omeprazole sulphone, and 20–4000 nmol/L for 5-hydroxyesomeprazole. The extraction recoveries ranged between 80 and 105%. The intra- and inter-day imprecision were less than 9.5% with accuracy between 97.7% and 100.1% for all analytes.
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*Keywords:* LC-MS/MS; Esomeprazole; Metabolites

## 1. Introduction

Esomeprazole (Fig. 1) is the first proton pump inhibitor (PPI) developed as an optical isomer (S-omeprazole) for the treatment of acid-related diseases [1]. Esomeprazole is a potent inhibitor of gastric acid secretion and accumulates in the acidic compartment of the parietal cells where the molecule is transformed to its active sulfenamide form. Esomeprazole is metabolized to two major metabolites, 5-hydroxyesomeprazole and esomeprazole sulphone (Fig. 1) [2].

Esomeprazole does not undergo chiral inversion in vivo [2] and therefore esomeprazole can be determined using the same methodology as for its racemate, omeprazole. Omeprazole has been determined in blood plasma by liquid chromatography with UV-detection [3–5] and this technique has also been employed for simultaneous assay of the two major metabolites [6]. In recent years the combination of liquid chromatography and mass spectrometry (LC-MS) has been used for omeprazole and metabolites [7–10] and for omeprazole and other PPIs [11,12]. Enantioselective methods for omeprazole by liquid chromatography have also been presented which employ mass spectrometric [13] or UV-detection [14]. Reported methods for omeprazole and its two major metabolites require a sample volume of 0.2–1.0 mL and a chromatographic run time of 16–60 min. In this paper we present a method based on LC-MS/MS after liquid–liquid extraction of esomeprazole and the two major metabolites using a plasma volume of 25 μL from human, rat or dog and a total liquid-chromatographic run time of about 6 min. The small sample volume has made the method adequate for toxicokinetic evaluation in rat and dog puppies and for pharmacokinetic evaluation in children (pre-term and neonates).

## 2. Experimental

### 2.1. Materials

Acetonitrile, dichloromethane, methyl *tert*-butyl ether (MTBE) and methanol were of HPLC grade from Rathburn Chemicals Ltd. (Walkerburn, Scotland). Ammonium acetate, formic acid, $NaH_2PO_4$, $Na_2CO_3$ and $NaHCO_3$ were of analytical grade from Merck KGaA (Darmstadt, Germany).

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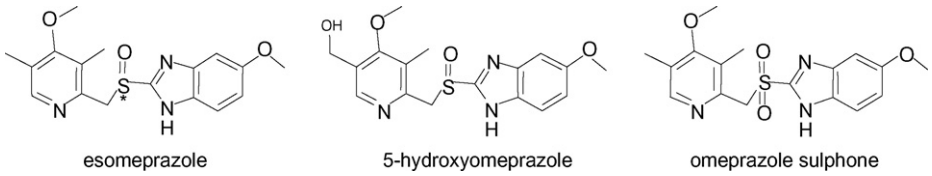


Fig. 1. Chemical structures of esomeprazole, 5-hydroxyomeprazole and omeprazole sulphone.

Esomeprazole magnesium trihydrate was obtained within AstraZeneca R&D (Södertälje, Sweden). Omeprazole sulphone (5′-methoxy-2′-[[(4-methoxy-3,5-dimethyl-2-pyridinyl) methyl]sulfonyl]-1*H*-benzimidazole) was obtained from Medicinal Chemistry (AstraZeneca R&D Mölndal, Sweden). Racemic 5-hydroxyomeprazole (*rac*-5′-methoxy-2′-[[(4-methoxy-3-methyl-5-hydroxymethyl- 2 -pyridinyl)methyl]sulfonyl]-1*H*-benzimidazole) was used as reference substance for 5-hydroxyesomeprazole and was obtained from Synthelec AB (Lund, Sweden). The internal standards omeprazole-$^{13}C_7$, 5-hydroxyomeprazole-$^{13}C_7$ and omeprazole sulphone-$^{13}C_7$ were obtained within AstraZeneca R&D Mölndal.

### 2.2. Calibration standards and quality control samples

Stock solutions of esomeprazole and omeprazole sulphone, at 50 μmol/L, and 5-hydroxyomeprazole, at 25 μmol/L, were prepared in 20% methanol in carbonate buffer (pH 9.3, 75 mmol/L). High working standard solutions were prepared from the three stock solutions and diluted with 20% methanol in carbonate buffer (pH 9.3, 75 mmol/L) to a concentration of 10 μmol/L of esomeprazole and omeprazole sulphone and 2 μmol/L of 5-hydroxyomeprazole. Low working standard solutions were prepared at a concentration of 10 nmol/L of each of esomeprazole, 5-hydroxyomeprazole and omeprazole sulphone. All standard solutions were stored at −20 °C.

Plasma standards were freshly prepared for each analytical batch of plasma samples at two concentration levels, 20 μmol/L (4 μmol/L for 5-hydroxyesomeprazole) and 20 nmol/L by adding 50 μL of the working solutions to 25 μL of drug-free human heparin plasma.

Stock solutions of each of the internal standards were prepared at 30 μmol/L in 20% methanol in carbonate buffer (pH 9.3, $I = 0.1$) and a working internal standard solution at 1 μmol/L of each by mixing and diluting with the same methanol aqueous solution.

Quality control samples at 60, 630 and 16,000 nmol/L (60, 280 and 3200 nmol/L for 5-hydroxyesomeprazole), were prepared in bulk from stock solutions, or dilutions thereof, by addition to drug-free human, rat or dog plasma. For the precision and accuracy experiments, test samples at 20 nmol/L and 20,000 nmol/L (4000 nmol/L for 5-hydroxyesomeprazole) were prepared. A maximum of 5% dilution of sample matrix was allowed. The bulk samples were aliquoted (0.2 mL) into polypropylene tubes and stored at −20 °C.

### 2.3. Sample preparation

Twenty-five microliter human blood plasma or 25 μL plasma from dog or rat combined with 75 μL drug-free human plasma was transferred to 5-mL glass tubes and mixed with 50 μL 20% methanol in carbonate buffer pH 9.3, 0.1 mol/L, 50 μL working internal standard solution and 25 μL 1 M $NaH_2PO_4$. This mixture had a pH of 6.0–6.7 and was extracted with 2 mL methyl *tert*-butyl ether-dichloromethane (3:2, v/v) on a mixing-plate (240 rpm) for 15 min and then centrifuged at 2200 g for 5 min. After freezing of the aqueous phase, by placing the tubes in a bath containing a mixture of ethanol and dry-ice, the organic phase was transferred to a new centrifuge tube and evaporated to dryness under nitrogen flow at 25 °C. The residues were dissolved in 500 μL 20% methanol in carbonate buffer (pH 9.3, 0.1 mol/L) by vortex-mixing (1 min), ultra-sonication (1 min) and vortex-mixing (1 min), and 15 μL was injected on the HPLC column.

Plasma blanks and quality control (QC) samples were assayed as above for the authentic samples. Plasma standards were assayed as above except for the addition of 50 μL 20% methanol in carbonate buffer.

### 2.4. Chromatographic conditions

The chromatographic system consisted of a Perkin-Elmer, 200 series pump and auto sampler (Wellesley, MA, USA). The separation was performed on a Hypersil BDS C8 separation column (50 mm × 4.6 mm, 3 μm) from Thermo Hypersil-Keystone (Waltham, MA, USA) using an Optiguard CN (15 × 1 mm) guard column from Optimize Technologies Inc. (Oregon City, OR, USA). The mobile phase was made by mixing 250 mL acetonitrile, 1.0 mL formic acid, 100 mL 0.1 mol/L ammonium acetate and 645 mL water (pH 3.8), and was pumped at a flow rate of 0.75 mL/min.

### 2.5. Mass spectrometric conditions

The effluent from the chromatographic column was split in a Valco split connection so that the liquid flow to the electro spray ion source of an API 3000 triple-quadrupole mass spectrometer with turbo-ion spray interface from MDS Sciex (Concord, ON, Canada) was about 150 μL/min. During the first 1.1 min the effluent from the chromatographic column was led directly to waste using a switching valve. The mass spectrometer was run in positive multiple-reaction-monitoring (MRM) mode with an ion source temperature of 475 °C, capillary voltage of 1.5 kV and with nitrogen collision gas at 5. Data acquisition and analysis were carried out using Analyst version 1.2 or 1.4.1 software.

### 2.6. Daily calibration

Calibration was made using two-point linear regression. With each analytical batch 6 plasma standards at each of 20 nmol/L,

Table 1
Extraction recovery

| Analyte | Concentration (nmol/L) | Human plasma | | Rat plasma | |
|---|---|---|---|---|---|
| | | Recovery (%) ($n=3$) | CV (%) | Recovery (%) ($n=3$) | CV (%) |
| Esomeprazole | 62 | 91.0 | 2.8 | 88.9 | 2.6 |
| | 620 | 88.6 | 2.9 | 89.3 | 2.4 |
| | 20000 | 88.8 | 0.3 | 89.0 | 1.6 |
| 5-Hydroxyesomeprazole | 61 | 82.7 | 7.5 | 79.1 | 9.0 |
| | 270 | 82.9 | 4.3 | 80.3 | 5.0 |
| | 4400 | 81.2 | 3.1 | 82.3 | 1.5 |
| Omeprazole sulphone | 66 | 100.1 | 1.5 | 97.4 | 3.7 |
| | 650 | 97.2 | 1.5 | 95.1 | 1.8 |
| | 20000 | 96.3 | 1.4 | 96.0 | 1.5 |

lower limit of quantification (LLOQ), and 20,000 nmol/L (4000 nmol/L for 5-hydroxyomeprazole), upper limit of quantification (ULOQ) were run.

### 2.7. Stability

Stability of the compounds in stock solutions and incurred samples were investigated. After storage, solutions were compared against freshly prepared solutions by direct injections on the LC-MS/MS system. Incurred samples were reassayed after storage at −18 °C for 6 or 12 months. The integrity of processed incurred samples was investigated by reinjection of samples after 72 h storage at room temperature.

## 3. Results and discussion

### 3.1. Extraction and separation

Esomeprazole, the sulphone and the hydroxymetabolite were isolated from blood plasma by liquid–liquid extraction to a solvent mixture of methyl *tert*-butyl ether and dichloromethane. Phase separation with an upper organic phase was achieved by centrifugation, and after freezing of the aqueous phase, the organic phase could easily be decanted to a new centrifuge tube and evaporated. Absolute extraction recovery from plasma was about 90% for esomeprazole, 80% for 5-hydroxyesomeprazole and 95% for omeprazole sulphone measured relative to a sam-

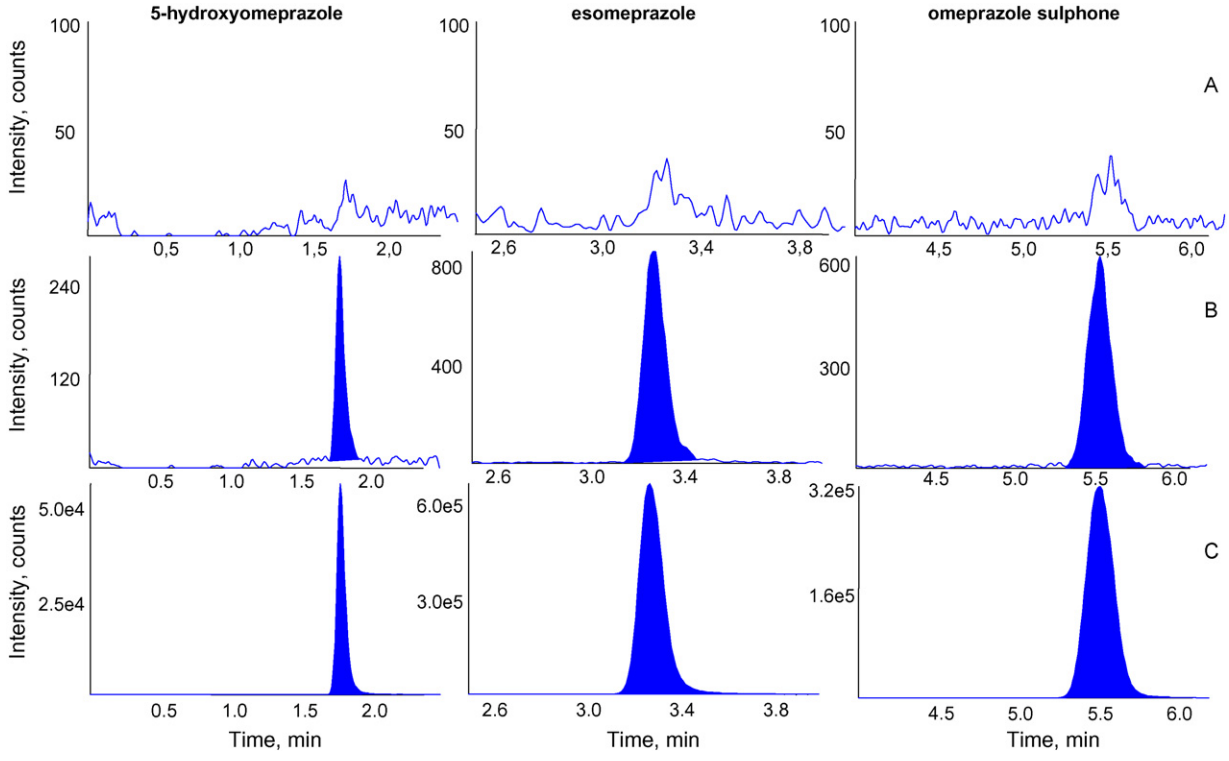


Fig. 2. Chromatograms for esomeprazole (transition 346 => 198 *m/z*), 5-hydroxyomeprazole (transition 362 => 214 *m/z*) and omeprazole sulphone (transition 362 => 150 *m/z*) from (A) a drug free plasma sample, (B) a plasma sample spiked at LLOQ and (C) a plasma sample spiked at ULOQ.

Table 2
Precision and accuracy (human)

| Analyte | Nominal concentration (nmol/L) | Intermediate precision ($n=9$) (%) | Repeatability ($n=9$) (%) | Accuracy ($n=9$) (%) |
|---|---|---|---|---|
| Esomeprazole | 21.8 | 7.6 | 6.7 | 103.3 |
| | 63.9 | 3.5 | 3.3 | 102.5 |
| | 20600 | 4.5 | 4.5 | 105.5 |
| 5-Hydroxyesomeprazole | 18.8 | 7.1 | 7.1 | 101.0 |
| | 55.1 | 4.8 | 4.8 | 100.2 |
| | 4100 | 4.4 | 4.4 | 105.2 |
| Omeprazole sulphone | 21.9 | 5.7 | 4.9 | 107.5 |
| | 64.3 | 4.6 | 4.6 | 101.5 |
| | 20500 | 4.4 | 4.4 | 102.0 |

ple with each substance added to extracted drug-free plasma (see Table 1). The extraction recoveries were independent of species and concentration, three levels being tested, and were of the same magnitude for the respective internal standards. Liquid–liquid extraction of omeprazole, the sulphone and the hydroxymetabolite using dichloromethane alone or in combination with acetonitrile or diethyl ether have previously been described [3,9,10]. The combination of methyl *tert*-butyl ether and dichloromethane (3:2) gives the advantage of an upper organic phase still keeping good recovery of the three analytes.

Liquid chromatographic separation was made on a Hypersil BDS C8 stationary phase with a mobile phase of acetonitrile in an aqueous solution containing ammonium acetate (10 mmol/L) and formic acid (0.1%). Chromatographic run time was 6.2 min, giving an injection-to-injection cycle time of 7.0 min. Chromatograms from a drug-free human plasma sample, a human plasma sample spiked at 20 nmol/L of all three analytes and a human plasma sample spiked at 20,000 of esomeprazole and omeprazole sulphone and 4000 nmol/L of 5-hydroxyesomeprazole, are shown in Fig. 2.

### 3.2. Selectivity

The selectivity was investigated by preparing and analyzing six individual human, three individual rat and three individual dog blank plasma samples. No interfering peaks were seen in either of the biological samples. A typical chromatogram from human plasma is shown in Fig. 2.

The sample matrix effect on ionization was determined by comparing the peak areas for esomeprazole, 5-hydroxyomeprazole and omeprazole sulphone (corresponding to a sample concentration at ULOQ) added to extracted drug-free plasma samples with those for the compounds added to reconstitution buffer at the same concentration. The effect was determined using six different blank samples from man, three from dog and three from rat. No significant sample matrix effect on ionisation was observed for either esomeprazole, 5-hydroxyesomeprazole or omeprazole sulphone as the response

Table 4
Check of linearity for esomeprazole in human plasma ($n=4$)

| | Nominal concentration (nmol/L) | | | | | |
|---|---|---|---|---|---|---|
| | 18.5 | 55.4 | 277 | 1110 | 6650 | 20000 |
| Mean | 17.4 | 54.6 | 264 | 1070 | 6440 | 19200 |
| CV (%) | 3.3 | 0.6 | 3.0 | 1.2 | 0.3 | 0.9 |
| Accuracy (%) | 94.1 | 98.6 | 95.2 | 96.5 | 96.8 | 96.0 |

Table 5
Check of linearity for 5-hydroxyesomeprazole in human plasma samples ($n=4$)

| | Nominal concentration (nmol/L) | | | | | |
|---|---|---|---|---|---|---|
| | 18.6 | 55.9 | 223 | 503 | 1340 | 4020 |
| Mean | 17.7 | 55.8 | 214 | 468 | 1290 | 3860 |
| CV (%) | 6.6 | 2.4 | 1.0 | 1.0 | 0.5 | 1.2 |
| Accuracy (%) | 95.3 | 99.9 | 96.0 | 93.0 | 95.9 | 96.0 |

Table 6
Check of linearity for omeprazole sulphone in human plasma samples ($n=4$)

| | Nominal concentration (nmol/L) | | | | | |
|---|---|---|---|---|---|---|
| | 18.7 | 56.0 | 280 | 1120 | 6710 | 20100 |
| Mean | 17.4 | 53.6 | 267 | 1090 | 6520 | 19600 |
| CV (%) | 2.8 | 1.8 | 3.2 | 0.5 | 0.1 | 0.8 |
| Accuracy (%) | 92.9 | 95.7 | 95.3 | 97.3 | 97.2 | 97.4 |

Table 3
Slopes and intercepts (with standard errors) for calibration equations from the three analytical batches used for estimation of precision and accuracy (each batch contained 12 plasma standards)

| Batch no. | Esomeprazole | | | 5-Hydroxyesomeprazole | | | Omeprazole sulphone | | |
|---|---|---|---|---|---|---|---|---|---|
| | Slope (SE) | Intercept (SE) | $r$ | Slope (SE) | Intercept (SE) | $r$ | Slope (SE) | Intercept (SE) | $r$ |
| 1 | 2.37 (0.0101) | 0.00361 (0.0607) | 0.9997 | 0.971 (0.00307) | 0.000815 (0.00509) | 0.9998 | 1.06 (0.00141) | 0.000383 (0.00928) | 1.0000 |
| 2 | 2.40 (0.0119) | 0.00235 (0.0714) | 0.9998 | 0.980 (0.00569) | 0.000087 (0.00860) | 0.9997 | 1.07 (0.00191) | 0.000045 (0.0126) | 1.0000 |
| 3 | 2.49 (0.0145) | 0.00605 (0.0873) | 0.9997 | 0.988 (0.00591) | 0.000765 (0.00893) | 0.9996 | 1.08 (0.00607) | 0.000659 (0.03990) | 0.9997 |

Table 7
Stability and processed sample integrity

| | Matrix | Time | Esomeprazole | | | 5-Hydroxy esomeprazole | | | Omeprazole sulfon | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Concentration (nmol/L) | Accuracy (%) | CV (%) | Concentration (nmol/L) | Accuracy (%) | CV (%) | Concentration (nmol/L) | Accuracy (%) | CV (%) |
| Authentic plasma samples (−18 °C) | Human | 12 months | 111–4100 | 94.6 ($n$ = 20) | 5.2 | 20.7–196 | 96.6 ($n$ = 20) | 9.8 | 33.6–1210 | 95.8 ($n$ = 20) | 4.7 |
| | Dog | 6 months | 29.2–16300 | 102.6 ($n$ = 25) | 10.0 | ND | ND | ND | 159–18100 | 105.4 ($n$ = 25) | 10.0 |
| | Rat | 6 months | 36.7–17800 | 97.9 ($n$ = 24) | 12.2 | ND | ND | ND | 43.3–18900 | 98.2 ($n$ = 22) | 8.9 |
| Processed authentic plasma samples (room temperature) | Human | 72 h | 31.8–4040 | 99.2 ($n$ = 26) | 1.4 | 30.9–427 | 102.0 ($n$ = 24) | 4.3 | 44.3–1970 | 101.6 ($n$ = 28) | 1.5 |
| | Dog | 72 h | 33.9–16500 | 100.1 ($n$ = 25) | 1.7 | 23.3–2430 | 101.7 ($n$ = 23) | 2.5 | 163–19300 | 100.0 ($n$ = 25) | 1.5 |
| | Rat | 72 h | 37.2–19200 | 100.7 ($n$ = 24) | 1.4 | 35.9–3690 | 101.8 ($n$ = 19) | 2.5 | 43.3–18900 | 100.9 ($n$ = 22) | 2.5 |
| Stock solution (−18 °C) | 20% Methanol in carbonate buffer (pH 9.3) | 12 months | 52000 | 100.1 | | 23000 | 103.7 | | 55000 | 98.1 | |
| Stock solution (room temperature) | 20% Methanol in carbonate buffer (pH 9.3) | 6 h | 52000 | 99.4 | | 25000 | 97.0 | | 50000 | 101.0 | |

relative to reconstitution buffer was 102.1–104.7% for human, rat and dog plasma.

### 3.3. Accuracy, precision and linearity

Accuracy, repeatability and intermediate precision for analysis of esomeprazole, 5-hydroxyesomeprazole and omeprazole sulphone at three concentrations in spiked human, rat and dog plasma samples were determined in three analytical runs. Acceptable accuracy and precision for all three analytes determined in human, rat and dog plasma samples were obtained as shown in Table 2 for human plasma, data from rat and dog were of the same quality. Equations for the calibrabation lines for the three analytical batches used to determine accuracy and precision can be found in Table 3. The method showed linearity over the calibration range of 20–20,000 nmol/L with acceptable accuracy and precision at the different concentration levels (Tables 4–6).

### 3.4. Stability

Stability of esomeprazole and the two metabolites in standard solutions, in plasma samples for long-term storage in freezer and integrity of processed samples for short-term storage in the auto sampler are shown in Table 7.

## 4. Conclusion

An analytical method was successfully developed with a sample preparation that had the advantage of an upper organic phase in the liquid–liquid extraction. The high recovery together with the sensitive detection made it possible to use small sample volumes. Due to the small sample volumes needed, and the relatively short chromatographic run time, this method have successfully been used for determination of esomeprazole, 5-hydroxyesomeprazole and omeprazole sulphone in plasma samples, from preclinical safety studies and from clinical pediatric studies, where esomeprazole has been administered and sample volumes have been limited.

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