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# Development of a LC–MS/MS-based method for determining metolazone concentrations in human plasma: Application to a pharmacokinetic study

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ABSTRACT

In this study, a method was developed and validated for the quantification of metolazone in human plasma samples. This method involves high-performance liquid chromatography coupled with tandem mass spectrometry and is more sensitive, selective and rapid than currently available methods. Chromatography was performed using a Phenomenex® Luna C18 column (100 mm × 2.0 mm, 5 μm, 100 Å) with an isocratic mobile phase of 0.1% formic acid:acetonitrile (40:60, v/v) and zaleplon as an internal standard. The drug and internal standard were extracted by liquid–liquid extraction and analyzed by mass spectrometry in the multiple reaction monitoring mode by using *m/z* values of 366.20/259.10 for metolazone and 306.20/235.60 for zaleplon. The calibration curve was linear over metolazone concentrations ranging from 0.02 ng/mL to 15 ng/mL. The lower limit of quantification was 0.02 ng/mL. Intra- and inter-assay precisions were 0.9–4.8% and 4.2–6.3%, respectively. The intra- and inter-assay accuracies in quantifying metolazone were 97.5–102.3% and 99.2–104.0%, respectively. Metolazone and zaleplon were eluted within 3.6 minutes, and the retention time was 1.75 minutes for metolazone and zaleplon. The validated method was successfully applied to a pharmacokinetic study of metolazone in human plasma.



## 1. Introduction

Metolazone (2-methyl-3-o-tolyl-6-sulphamyl-7-chloro-1,2,3,4-tetrahydro-4-quinazolinone) is a diuretic, saluretic and antihypertensive drug of the quinazoline class with therapeutic characteristics comparable to those of thiazide diuretics [1–3]. Metolazone inhibits sodium reabsorption primarily at the cortical diluting site in the renal tubules. This occurs to a lesser extent in the proximal convoluted tubule. The diuretic activity of metolazone relates to the rate and extent of its urinary delivery, which in turn is linked to its plasma content and indirectly to its concentrations in whole blood [4–8].

Metolazone is incompletely absorbed by the gastrointestinal tract; the rate and extent of absorption depend on its formulation. Approximately 95% of metolazone is in the bound form in circulating blood, that is, 50–70% is bound to red blood cells and 15–33% to plasma proteins. Its diuretic effects persist for 24 hours [1–7].

The therapeutic doses of Mykrox® (metolazone, 0.5 mg) recommended for patients are low (0.5–1.0 mg) and result in

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low therapeutic concentrations in biological fluids [5]. Therefore, a sensitive method is required. Several analytical methods for the determination of metolazone concentrations in biological fluids, including plasma, whole blood and urine, are available. Existing analytical methods for detecting metolazone include high-performance liquid chromatography (HPLC) systems equipped with detectors using ultraviolet radiation, fluorescence and mass spectrometry [6,7,9–12]. HPLC methods have certain limitations and are not sensitive enough for exact determination of terminal half-life in pharmacokinetic studies involving lower doses (0.5 mg). Recently, Jia et al. developed a liquid chromatography coupled with tandem mass spectrometry (LC–MS/MS) method for the quantitative determination of metolazone with a lower limit of quantification (LLOQ) of 0.05 ng/mL. However, it is still necessary to develop a more sensitive method to measure the concentrations of metolazone in human plasma for advanced pharmacokinetic profiles in low dose metolazone [10–12].

The purpose of the present study was to develop a simpler and more rapid and sensitive assay for the quantitative determination of metolazone in plasma samples. LC–MS/MS systems are some of the most powerful analytical tools in clinical pharmacokinetics owing to their selectivity, sensitivity and reproducibility, and have been validated for linearity, precision and accuracy. The method described in the present study was applied to a pharmacokinetic study. Pharmacokinetic studies of metolazone have rarely been reported and our present study provides pharmacokinetic data for future reference.

## 2. Methods

### 2.1. Chemicals

USP Reference Standard metolazone was purchased from the United States Pharmacopeial Convention (Rockville, MD, USA). Zaleplon (internal standard; 99.2%) was obtained from Adamed Sp.zo.o (Warsaw, Poland). HPLC-grade acetonitrile was purchased from Mallinckrodt Baker, Inc. (Phillipsburg, NJ, USA). Analytical grade ammonium acetate, dipotassium hydrogen phosphate, formic acid and acetic acid were purchased from Merck KGaA (Darmstadt, Germany). Reagent-grade diethyl ether was purchased from J.T. Baker (Phillipsburg, NJ, USA). HPLC-grade water was prepared using a Milli-Q system (EMD Millipore Corp., Billerica, MA, USA).

### 2.2. Instrumentation

The HPLC system (1100 Series; Agilent Technologies, Wilmington, DE, USA) was equipped with an MS/MS detection system consisting of an API 4000 model (Applied Biosystems, Foster City, CA, USA). Data processing was performed with Analyst 1.4 software (AB SCIEX, Framingham, MA, USA).

### 2.3. Chromatographic conditions

A Phenomenex® Luna C18 column (100 mm × 2.0 mm, 5 μm, 100 Å) was selected as the analytical column. The mobile phase was 0.1% formic acid:acetonitrile (40:60, v/v). The flow rate of the mobile phase was 0.4 mL/min, the injection volume was 10 μL, and the column temperature was 35 °C.

Electrospray ionization (ESI) was used in the positive ion detection mode. The capillary temperature was maintained at 500 °C, the spray voltage was 4.5 kV, and the collision-activated dissociation of the nitrogen gas was 5.0. The instrument was set up in multiple-reaction monitoring mode and the transition *m*/z values were 366.20/259.10 for metolazone and 306.20/235.60 for zaleplon.

### 2.4. Preparation of calibration standards and quality control samples

A stock solution of metolazone (100 μg/mL) was prepared by dissolving metolazone in acetonitrile:water (50:50, v/v). Subsequently, the working solution was serially diluted (1–10,000 ng/mL) with acetonitrile:water (50:50, v/v). A calibration curve was prepared using different concentrations of working stock solutions. The calibration curves of metolazone were prepared at the concentration levels of 0.02, 0.05, 0.1, 0.3, 1, 5, 10 and 15 ng/mL. The quality control (QC) samples were prepared at concentrations of 0.02 ng/mL (LLOQ), 0.06 ng/mL [low QC (LQC)], 0.6 ng/mL [medium QC (MQC)], and 12 ng/mL [high QC (HQC)] with blank plasma, by separate weighing of a reference standard. An internal standard of zaleplon (100 ng/mL) was prepared by diluting a stock solution with acetonitrile:water (50:50, v/v). The calibration standards were prepared fresh and the QC samples were stored in the freezer at −20 °C until analysis. All the solutions were stored at 2–8 °C and brought to room temperature before use.

### 2.5. Sample preparation

Liquid–liquid extraction was used to isolate metolazone and zaleplon from human plasma. Briefly, 50 μL of zaleplon solution (100 ng/mL) was added to a 0.5-mL aliquot of plasma as the internal standard for LC–MS/MS analysis. Following the addition of 4 mL of diethyl ether, the samples were vortexed for 5 minutes and then centrifuged at 1900*g*. The organic layer was transferred to fresh tubes and evaporated to dryness under a stream of nitrogen. Residues were reconstituted in 200 μL of mobile phase, and a 10-μL aliquot was injected into the LC–MS/MS system.

### 2.6. Calibration curve, regression model, precision and accuracy

The analytical curves were constructed using values ranging from 0.02 ng/mL to 15 ng/mL in human plasma. Calibration curves were obtained by weighted (1/concentration) least-squares regression analysis. The ratio of the peak area of metolazone to that of zaleplon was plotted against metolazone concentrations (ng/mL). Calibration curve standards and QC samples were prepared for analysis in six replicates. Precision and accuracy for the interpolated concentrations of the calibration points were maintained within ±15% of their nominal values. However, for the LLOQ, the precision and accuracy were set within ±20%. The matrix test was performed to evaluate the suppression or enhancement of the ionization of analytes and the internal standard by the

presence of matrix components in biological samples. In the present study, 12 blank plasma samples from six different sources were prepared for the evaluation of matrix effect. Following the sample preparation method, samples were prepared to re-dissolve the solution step; 200 μL of 1.5 ng/mL of metolazone and 25 ng/mL of the internal standard were then added. After the analyses of all samples, the coefficients of variance were calculated and used to evaluate the significant influence of matrix effect.

### 2.7. LLOQ

The LLOQ was estimated in accordance with the baseline noise method. It was estimated at a signal-to-noise ratio of five. The LLOQ was experimentally determined by administering six injections of metolazone at the LLOQ concentration. The signal-to-noise ratio was calculated by selecting the noise region as close as possible to the signal peak, which was at least 10 times the width of the signal peak at half its height.

### 2.8. Recovery

The recovery of metolazone was evaluated by comparing the mean peak area of six extracted LQC, MQC and HQC samples and plasma samples with 0.06, 0.6 and 12 ng/mL metolazone concentrations with the mean peak area of six unprocessed samples (blank sample extracts spiked with working solution).

### 2.9. Stability (freeze–thaw, auto-sampler, long-term, short-term)

LQC and HQC samples ($n = 3$) were retrieved in triplicate from the freezer after four freeze–thaw cycles and were then frozen at −20 °C. The samples were thawed on the laboratory bench or in a water bath at room temperature (25 °C). Auto-sampler stability was studied following a 24-hour storage period in the auto-sampler tray. Long-term stability of metolazone in the QC samples was also determined after 114 days of storage at −20 °C. Short-term stability was determined for 24 hours with LQC and HQC samples. Stability samples were processed and extracted along with freshly spiked calibration curve standards.

### 2.10. Application of the method

The methods described above were applied in a pharmacokinetic study involving 20 healthy male Taiwanese volunteers (age range, 20–40 years). They were free from cardiac, hepatic, renal, pulmonary, neurological, gastrointestinal and hematological diseases, as assessed by physical examination, electrocardiography analysis and laboratory analyses, including hematology, biochemistry and electrolyte studies and urinalysis. All subjects were instructed to abstain from taking any medication for 2 weeks prior to and during the entire study. The study protocol was approved by the Institutional Review Board of Mackay Memorial Hospital in accordance with the principles of the Declaration of Helsinki. Written informed consents were obtained from all the subjects. The study was conducted according to a single-center open-label design. The study subjects fasted overnight and were then administered a single oral dose of 0.5 mg metolazone tablets with 240 mL of water. Mykrox® tablets were used as an investigation drug. Standardized meals were served 4 and 10 hours after dosing. Venous blood samples (10 mL) were drawn in heparinized tubes before dosing (0 hours) and at 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 6.0, 8.0, 10, 12, 24, 36 and 48 hours after drug administration. Plasma samples were immediately separated by centrifugation at 1900*g*. All samples were stored at −20 °C until analysis.

### 2.11. Pharmacokinetic analysis

A non-compartmental pharmacokinetic method was used to determine the pharmacokinetic parameters. $C_{max}$, the maximum observed concentration, and $T_{max}$, the time to peak concentration, were determined for each subject and treatment. The area under the concentration–time curve from time 0 to the last quantifiable concentration ($AUC_{0-t}$) was determined by the trapezoidal rule. The area under the concentration–time curve from time 0 to infinity ($AUC_{0-\infty}$) was determined by the trapezoidal rule and extrapolated to infinity, as estimated by the last quantifiable concentration divided by the elimination rate constant ($k_{el}$). The $k_{el}$ was determined by simple linear regression based on the terminal phase of plasma concentration. Plasma half-life ($T_{1/2}$) was estimated using the following calculation: $0.693/k_{el}$.

## 3. Results and discussion

### 3.1. Chromatography

Chromatographic conditions were optimized in several trials to achieve good resolution and symmetrical peak shapes. The chromatograms of extracted human plasma samples are shown in Fig. 1. No significant interfering peaks from endogenous components were observed in blank plasma (Fig. 1A). The chromatographic retention time was 1.75 minutes for metolazone and zaleplon with a total run time of 3.6 minutes. Our metolazone retention time is much shorter than the times of 6.3 minutes [10] and 3.7 minutes [12] reported previously. The results suggest that the LC–MS/MS assay system is fast and suitable for the analysis of metolazone in plasma samples.

### 3.2. Linearity, precision, and accuracy

The calibration curve for the ratio of peak area–concentration of zaleplon versus that of metolazone showed a linear relationship over a concentration range of 0.02 ng/mL to 15 ng/mL. The regression equation of metolazone was $y = 0.6888x + 0.000414$, and the correlation coefficient ($r^2$) was 0.9998, indicating good linearity. Deviation from the expected concentration ranged from 97.4% to 102.0%, and the coefficients of variation were all within 4% (Table 1).

Roy et al. also developed an LC–MS/MS method using ESI+ tandem mass spectrometry for quantifying metolazone in human plasma [11]. Jia et al. reported the determination of metolazone in human plasma by liquid chromatography with

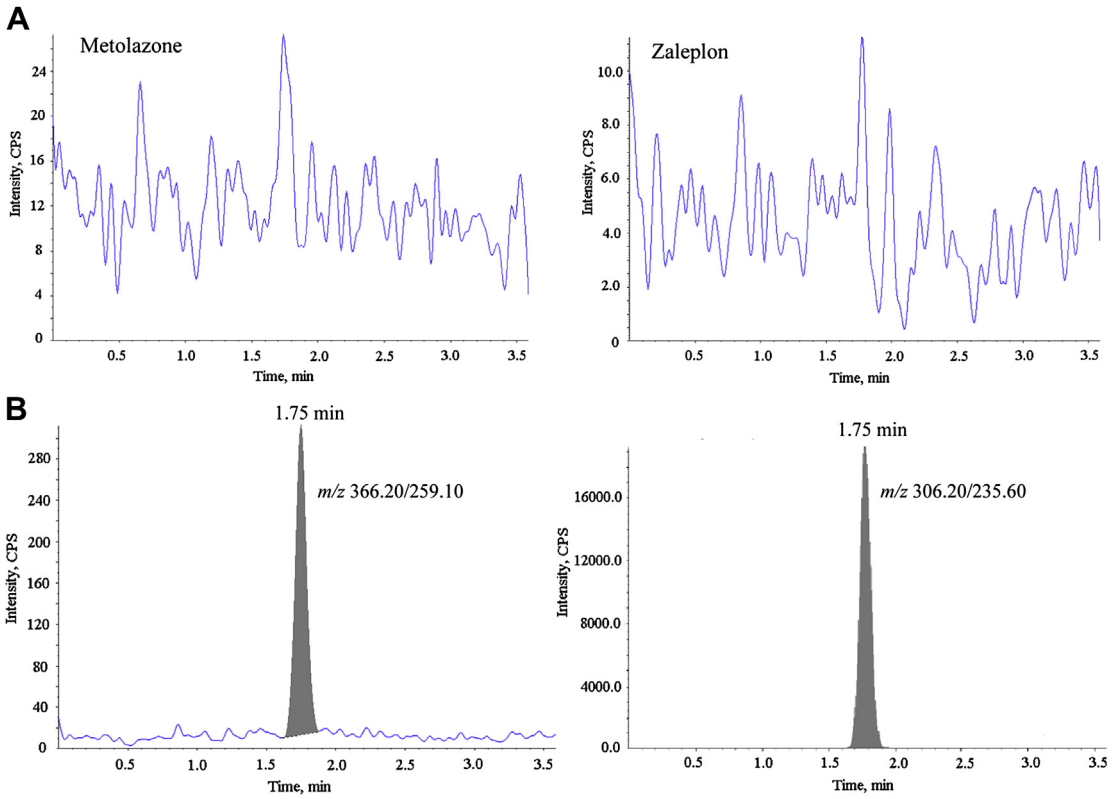


**Fig. 1 – Chromatograms of (A) drug-free plasma sample and (B) plasma sample spiked with 0.02 ng/mL metolazone (lower limit of quantification) and 10 ng/mL of zaleplon (internal standard).**

ESI–tandem mass spectrometry [12]. The LLOQ values of 2 ng/mL and 0.05 ng/mL reported in these two studies, which are much higher than those obtained in the present study, indicate that the sensitivity of the present method is sufficient for bioanalysis.

Precision and accuracy were determined at four different concentrations on six independent occasions. Table 2 shows that the intra-assay coefficients of variation ranged from 0.9% to 4.8%, and trueness ranged from 97.5% to 102.3%. The inter-assay coefficients of variation ranged from 4.2% to 6.3%, and trueness ranged from 99.2% to 104.0%, indicating that the assay method was sufficiently reliable and reproducible within the required analytical range. Based on the results of the matrix effect test, the coefficient of variation of 12 plasma samples from six different sources was 1.3%. The results indicated that the matrix component in human plasma did not significantly affect response.

### 3.3. *Recovery*

The recoveries of metolazone when added to plasma at concentrations of 0.06, 0.60 and 12.0 ng/mL were 95.2%, 88.6% and 87.2%, respectively. The average recoveries of metolazone and zaleplon, that is, 90.3% and 90.4%, respectively, were judged to

**Table 1 – Precision and accuracy of assays for plasma spiked with metolazone.**

| Spiked concentration (ng/mL) | Back-calculated concentration (ng/mL) |
|---|---|
| 0.02 | 0.0203 ± 0.0008 (3.9) |
| 0.05 | 0.0491 ± 0.0013 (2.6) |
| 0.1 | 0.100 ± 0.002 (2.0) |
| 0.3 | 0.306 ± 0.006 (2.0) |
| 1 | 1.01 ± 0.2 (2.0) |
| 5 | 4.82 ± 0.03 (0.6) |
| 10 | 10.1 ± 0.2 (2.0) |
| 15 | 15.0 ± 0.2 (1.3) |

Data are expressed as mean ± standard deviation (coefficient of variation %), $n = 5$.

**Table 2 – Precision and accuracy of assays for detecting metolazone in human plasma.**

| | Intra-assay ($n = 5$) | | | | Inter-assay ($n = 30$) | | | |
|---|---|---|---|---|---|---|---|---|
| Metolazone (ng/mL) | 0.02 | 0.06 | 0.6 | 12 | 0.02 | 0.06 | 0.6 | 12 |
| Mean metolazone (ng/mL) | 0.0203 | 0.0608 | 0.614 | 11.7 | 0.0208 | 0.0622 | 0.609 | 11.9 |
| CV (%) | 3.4 | 4.8 | 3.9 | 0.9 | 4.8 | 6.3 | 4.4 | 4.2 |
| RE (%) | 1.5 | 1.3 | 2.3 | −2.5 | 4.0 | 3.7 | 1.5 | −0.8 |
| Trueness (%) | 101.5 | 101.3 | 102.3 | 97.5 | 104.0 | 103.7 | 101.5 | 99.2 |

CV = coefficient of variation; RE = relative error.

**Table 3 – Stability of metolazone in three human plasma samples.**

| Stability (mean of percentage remaining, %) | Metolazone concentration (ng/mL) | |
|---|---|---|
| | 0.06 | 12 |
| Short-term (24 h, room temperature) | 92.5% | 94.2% |
| Auto-sampler (24 h, room temperature) | 111.5% | 104.2% |
| Freeze–thaw in air (laboratory bench) (4 cycles) | 103.0% | 95.0% |
| Freeze–thaw in water bath (4 cycles) | 99.8% | 95.8% |
| Long-term storage at −20 °C (114 d) | 101.5% | 98.3% |

be acceptable for clinical testing. The recoveries of the present method exceeded the 69.8–74.7% [10] and 70.5–72.6% [12] reported previously, which may be due to the lower LLOQ of this bioanalytical method.

### 3.4. *Stability studies*

Table 3 summarizes the results of the stability studies and shows that the analytes were stable after plasma samples were subjected to four freeze–thaw cycles (−20 °C to 25 °C). Differences in metolazone concentrations after the fourth freeze–thaw cycle ranged from 95.0% to 103.0%. The results indicated that metolazone was stable in human plasma after four freeze–thaw cycles. No significant degradation was observed even after a 24-hour storage period in the auto-sampler tray, and the final concentrations of metolazone ranged from 104.2% to 111.5%. Moreover, the long-term stability of metolazone in the LQC and HQC samples after 114 days of storage at −20 °C was also evaluated, and metolazone concentrations ranged from 98.3% to 101.5%.

### 3.5. *Application to a pharmacokinetic study*

We next determined the pharmacokinetics of a single 0.5-mg dose (1 tablet) of metolazone in 20 healthy male volunteers. Fig. 2 shows a representative plot of plasma concentration as a function of time. Published LLOQs are not low enough to allow the determination of metolazone concentrations in human plasma following a 0.5 mg dose [10–12]. The LLOQ determined here was 0.02 ng/mL. Our present results showed that the

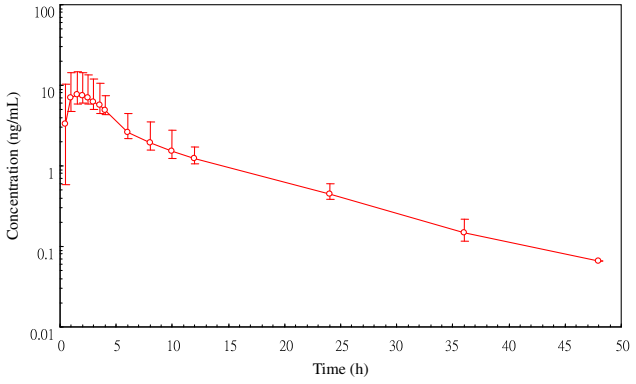


**Fig. 2 – Mean plasma concentration–time profile after oral administration of 0.5 mg metolazone tablets.**

**Table 4 – Pharmacokinetic parameters of metolazone following a single oral dose administered to healthy subjects in the present study and in Jia et al.'s study [12].**

| | Present study ($n = 20$) | Jia et al.'s study ($n = 10$) | | |
|---|---|---|---|---|
| | 0.5 mg | 0.5 mg | 1 mg | 2 mg |
| $AUC_{0-t}$ (h × ng/mL) | 56.5 ± 10.4 | 33.9 ± 9.2 | 122.5 ± 36.3 | 162.4 ± 26.9 |
| $AUC_{0-\infty}$ (h × ng/mL) | 57.3 ± 10.8 | 34.2 ± 8.9 | 123.9 ± 37.0 | 164.1 ± 27.5 |
| $C_{max}$ (ng/mL) | 8.71 ± 1.16 | 6.9 ± 2.6 | 20.6 ± 4.8 | 36.8 ± 7.1 |
| MRT (h) | 9.07 ± 1.34 | 5.7 ± 1.0 | 8.1 ± 1.3 | 6.8 ± 1.5 |
| $T_{max}$ (h) | 1.78 ± 0.77 | 1.55 ± 0.9 | 1.60 ± 0.6 | 1.45 ± 1.3 |
| $T_{1/2}$ (h) | 8.27 ± 0.86 | 6.6 ± 2.8 | 7.9 ± 1.2 | 6.8 ± 1.5 |

Data are expressed as mean ± standard deviation.

presented time–concentration profiles were clearer than those in human whole blood [10].

The pharmacokinetic characteristics are consistent with those of other studies administering 1–2 mg of metolazone in human plasma [10–12]. Compared to the published data on administering 0.5 mg of metolazone, the variations in pharmacokinetic parameters are higher, and the AUC values are smaller than those reported in the current study [12]. We conjecture that the difference might be due to the sensitivity of this bioanalytical method.

The plasma concentrations of metolazone were within the calibration curve range and remained above the 0.02 ng/mL sensitivity limit for the entire sampling period. $AUC_{0-t}$ and $AUC_{0-\infty}$ were 56.5 ± 10.4 h × ng/mL and 57.3 ± 10.8 h × ng/mL, respectively. $C_{max}$ was 8.71 ± 1.16 ng/mL and $T_{1/2}$ was 8.27 ± 0.86 hours. The mean ratio of $AUC_{0-t}/AUC_{0-\infty}$ was over 90%. All the pharmacokinetic parameters of metolazone are listed in Table 4.

In the present study, we could determine the concentrations of metolazone up to 48 hours after the oral administration of 0.5 mg to healthy volunteers. Thus, we could assess the drug's elimination phase by using a more efficient extraction method coupled with more sensitive analytical techniques. The methods developed in the present study are suitable for pharmacokinetic studies.

## 4. Conclusion

A simple, rapid and specific liquid chromatography tandem mass spectrometric method was developed and validated for the quantitative measurement of metolazone. It could be prepared by liquid–liquid extraction, separated by a Luna C18 column and quantified by LC–MS/MS. This method offers the advantages of higher sensitivity and shorter run time. It was successfully applied to characterize the pharmacokinetics of metolazone in healthy volunteers.

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