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Bioavailability measurements of methazolamide in plasma, red blood cells and whole blood: implications for bioequivalence studies

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Abstract

The kinetics of methazolamide (MTZ) in human whole blood, red blood cells (RBCs) and plasma have been investigated. The study was conducted over a wide dose range using three single-dose (25, 50 or 100 mg) and two multiple-dose (50 mg q 12 h × 3 or 100 mg q 12 h × 3) schedules. The results of the study indicate that the increase of drug concentrations in both whole blood and RBCs is dose-proportional over a range of 25 mg to 150 mg doses, while that in plasma is dose-proportional only from 100 mg to 150 mg. As plasma drug concentrations at 100 mg dose is too low to provide a reliable measure, and a dose higher than this may cause adverse reactions in healthy individuals, the pharmacokinetic parameters derived from whole blood analysis at the 100 mg dose is adequate for bioequivalence evaluation of the drug product obtained from two different sources.

Keywords: Methazolamide; Whole blood; Red blood cell; Plasma; Dose proportionality; Distribution; Pharmacokinetic parameters; Bioequivalence study

1. Introduction

With the development of generic drug industries, the new generic products need testing for their bioequivalency before they are marketed. Bioavailability studies are useful in evaluating a new drug (innovator product) in terms of its formulation effect on the pharmacokinetics of the drug, whereas bioequivalence studies are necessary to compare the relative bioavailabilities, ex-

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pressed in terms of rate and extent of absorption of two or more chemically or pharmaceutically equivalent drugs which produce comparable bioavailable characteristics in individuals when administered in equivalent dosage regimens. In general, bioequivalence studies submitted in advance of market approval to the Food and Drug Administration (FDA) are conducted by measuring plasma or serum drug concentrations over time. Although this approach is suitable for many drugs binding to plasma proteins, it becomes inappropriate for some drugs those bind extensively with tissue constituents, such as erythrocytes (red blood cells, RBCs). For drugs that bind strongly to RBCs, hematocrit plays an important role in defining the relative amount or concentration of the drug in the blood, RBCs and plasma. Furthermore, ratios of plasma to RBCs concentrations of some drugs are not constant because of non-linear binding to the RBCs as determined by dose-proportionality studies. Data obtained from methazolamide (MTZ) bioavailability studies submitted to the Food and Drug Administration illustrated these concepts and served as a basis for a retrospective analysis to determine whether whole blood concentrations are adequate to establish the bioequivalence of multi-source MTZ formulations. MTZ is a potent carbonic anhydrase (CA) inhibitor used in the treatment of glaucoma.

2. Materials and methods

2.1. Design of clinical study

Study participants were 17 non-institutionalized, male and female glaucoma patients with an average age of 52.3 years (range: 25–78 years). Study participants were divided into three groups (I, II, III) with five or six subjects per group. Group I received a single 25 mg dose of MTZ. Groups II and III received 50 mg q 12 h × 3 and 100 mg q 12 h × 3 doses, respectively.

The first and third doses were administered after an overnight fast, and no food was allowed for 2 h after dosing. Blood samples were collected into evacuated tubes containing heparin at 0 (predose) and 0.5, 1, 2, 3, 4, 6, 8, 10, 12 h after the administration of the first dose in all groups (17 subjects) and again after the third dose in Groups II and III. The second dose was given 12 h after the first dose, immediately after the last blood sample collection.

2.2. Analysis of drugs in plasma and whole blood

For drug analysis, the blood sample was divided into two portions: (1) one portion was processed to separate plasma and RBCs with analysis carried out in plasma; (2) the other portion was analyzed as whole blood. Hematocrits were determined. MTZ concentrations in whole blood and plasma were measured by high performance liquid chromatography (HPLC) with UV detection, a method similar to that described in Matuszewski et al. (1994). The assay methodology was validated to obtain acceptable linearity, sensitivity, precision and specificity. The practical limit for the detection of MTZ was 0.05 μg/ml. MTZ concentrations in RBC (C_{RBC}) were calculated according to the following equation:

$$C_{RBC} = \frac{C_{WB} - (1 - H)C_P}{H}$$

where C_{WB} and C_P are the measured concentrations of methazolamide in whole blood and plasma respectively, and H is the hematocrit (Bayne et al., 1981).

2.3. Analysis of data

Due to the risk of possible adverse reactions (Physician's Desk Reference, 1995), 150 mg and 300 mg doses were not administered as single doses, but were divided and given in three equal doses at 12-h intervals. As reported earlier by Bayne et al. (1981), methazolamide has an apparent half-life of 15 days in RBCs, and therefore, very little of the drug will be expected to be eliminated from the body in 12-h or 24-h periods after its administration. Consequently, these triple doses (50 mg q 12 h × 3 and 100 mg q 12 h × 3) may allow an estimate of the absorption and distribution of single equivalent doses of 150 mg and 300 mg of methazolamide in 24–36 h.

The following parameters for whole blood, RBC and plasma drug concentrations were calculated to assess bioavailability: (1) the area under the drug concentration-time curve from time zero to time of the last quantifiable concentration ($AUC_{0\text{-}T}$) and (2) the maximum observed drug concentration (C_{max}). $AUC_{0\text{-}T}$ was estimated by using the linear trapezoid method of Gibaldi and Perrier (1982).

3. Results and discussion

Single dose studies with 25 mg, 50 mg, and 100 mg doses of MTZ in the 17 study participants indicated that the mean drug concentrations in whole blood (Fig. 1A) and RBCs (Fig. 2A) increased proportionally over 12 h with dose. Because more than 95% of methazolamide in blood is strongly bound to RBCs (Maren, 1962, 1967; Coleman, 1975), the temporal pattern of RBC-bound drug concentration (Fig. 2A) followed that of the whole blood drug concentration profile (Fig. 1A). Also, because most of the drug is located within the RBC pool, the concentrations of drug in RBCs were noted to be about twice those of whole blood. In contrast, the temporal

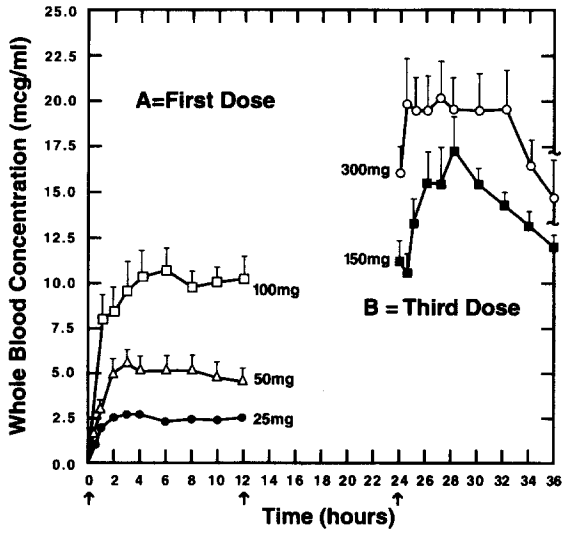

Fig. 1. Methazolamide concentrations (μg/ml) in whole blood of subjects treated with (A) single (25, 50, or 100 mg), and (B) triple (50 mg q 12 h × 3, or 100 mg q 12 h × 3) doses. Doses were given (at arrows, ↑) at 0, 12 and 24 h after taking blood samples. Each value represents the mean ± S.E. of five or six subjects of each group (some error bars are within symbols).

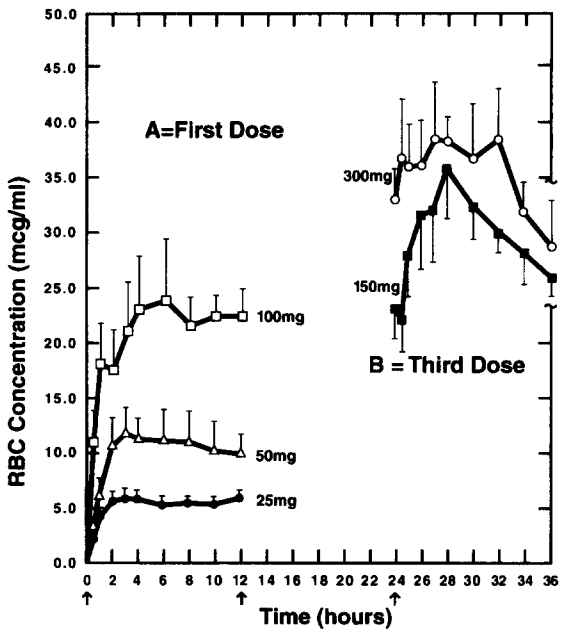

Fig. 2. Methazolamide concentrations (μg/ml) in RBCs of subjects treated with (A) single, and (B) triple doses as in Fig. 1 (other details are same as given for Fig. 1).

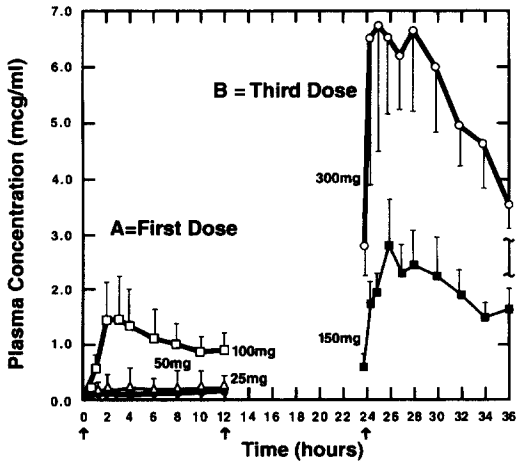

Fig. 3. Methazolamide concentrations (μg/ml) in plasma of subjects treated with (A) single (25, 50, or 100 mg), (B) triple (50 mg q 12 h × 3, or 100 mg q 12 h × 3) doses. Doses were given at arrows (↑) as in Fig. 1 (other details are same as given for Fig. 1).

Table 1
Linear and non-linear increase of whole blood, RBC and plasma drug levels 3 h after the dosage administered

Samples[a]	Dosages (mg)	Subjects	Methazolamide concentration (μg/ml) Mean ± S.D.	C.V.(%)[b]
Whole blood	25	6	2.45 ± 1.0	39
	50	6	5.33 ± 2.5	47
	100	5	9.62 ± 4.8	50
	150	6	15.47 ± 5.7	37
	300	5	20.30 ± 5.1	25
RBC	25	6	5.56 ± 2.4	44
	50	6	11.63 ± 5.7	49
	100	5	20.66 ± 10.7	52
	150	6	31.56 ± 11.6	37
	300	5	37.98 ± 11.3	30
Plasma	25	6	0.04 ± 0.03	74
	50	6	0.12 ± 0.04	32
	100	5	1.40 ± 1.61	115
	150	6	2.22 ± 1.22	55
	300	5	6.12 ± 2.18	26

[a]Data obtained from single (25, 50 or 100 mg) and triple (50 mg × 3, or 100 mg × 3, each dose given at 0, 12 and 24 h) dose studies.
[b]Coefficient of variation

patterns of plasma drug concentrations did not indicate dose-proportionality (Fig. 3A) and did not correspond well to whole blood level time profiles. Three hours after administration of the single 25 mg, 50 mg and 100 mg doses, the mean plasma concentrations were 0.04, 0.12 and 1.40 μg/ml, respectively. Three hours after the 150 mg dose, mean plasma concentration increased to 2.22 μg/ml (Table 1), an 18-fold increase in concentration corresponding to a 3-fold increase in dose (50–150 mg). This finding is in agreement with that reported by Bayne et al. (1981).

Data for the equivalent doses of 150 mg (50 mg q 12 h × 3) and 300 mg (100 mg q 12 h × 3) indicate that the increases of MTZ concentrations in RBCs and whole blood are dose-proportional only up to an equivalent dose of 150 mg (Fig. 1B and Fig. 2B). Dose-proportionality, however, was not observed at the equivalent dose of 300 mg. Bayne et al. (1981) noted that RBC saturation was not found after a single 150 mg dose, but was observed at a higher dose. As a result of saturable RBC binding, mean plasma concentration of MTZ at the equivalent 300 mg dose was also disproportionately higher than those of the lower doses (Fig. 3B).

The $AUC_{0\text{-}T}$ and C_{max} data after single 25 mg, 50 mg, and 100 mg doses of MTZ (Table 2) indicate that a 2-fold dose increase from 25 mg to 50 mg and from 50 mg to 100 mg was accompanied by a corresponding 2-fold increase in whole blood and RBC bioavailability. In contrast, an 8-fold increase in plasma $AUC_{0\text{-}T}$ or C_{max} was observed when dose was doubled from 50 mg to 100 mg, indicating a non-linear binding of MTZ to plasma proteins at this concentration range.

Coleman in 1975 reported that there is only one binding site per CA molecule (Coleman, 1975). Seven isozymes of CA have been reported, differing in physical and kinetic properties in susceptibility to inhibitors and in tissue-specific expression (Biollaz et al., 1995). CA in RBCs accounts for more than 90% of the enzyme in the body (Maren et al., 1977). Human RBCs have been reported to contain 156 μM of CA, of which 20 μM isoenzyme is of low capacity and high affinity type II and 136 μM isoenzyme is of high capacity and low affinity type I (Wistrand and Baathe, 1968). Extravascular carbonic anhydrase, including that found in the ciliary body, belongs to the type II category (Maren et al., 1976).

Table 2
Pharmacokinetic parameters, $AUC_{0\text{-}T}$ and C_{max} derived from whole blood, RBC and plasma drug concentrations

Samples[a,b]	Dosage (mg)	Subjects	$AUC_{0\text{-}T}$ (μg/h per ml) Mean ± S.D. (C.V.%)[c]	C_{max} (μg/ml) Mean ± S.D. (C.V.%)
Whole blood	25	6	26.0 ± 7.8 (30)	2.8 ± 0.8 (28)
	50	6	54.4 ± 26.8[d] (49)	6.3 ± 2.4[e] (38)
	100	5	113.1 ± 37.2[d] (33)	14.3 ± 4.3[e] (30)
RBC	25	6	59.2 ± 20.7 (34)	6.4 ± 2.2 (34)
	50	6	118.3 ± 59.8[d] (50)	13.7 ± 5.4[d] (39)
	100	5	245.8 ± 78.9[e] (32)	31.2 ± 10.1[e] (32)
Plasma	25	6	0.5 ± 0.3 (56)	0.1 ± 0.1 (92)
	50	6	1.4 ± 0.6[d] (41)	0.2 ± 0.1[f] (76)
	100	5	11.6 ± 11.6[g] (100)	1.5 ± 1.5[g] (99)

[a]Data obtained from single-dose studies.
[b]Assay conditions are described under Materials and methods.
[c]Coefficient of variation.
Probability level [e]$P < 0.005$; [d]$P < 0.025$; [g]$P < 0.05$; [f]$P > 0.05$.

MTZ has been shown to bind primarily to CA in the RBCs and the binding is of reversible nature (Maren, 1962). In the present study, the non-linear shape of the curve in Fig. 4 suggests that MTZ binds to more than one site on CA. Fig. 4 shows the binding curve relating the ratio of RBC-bound drug concentration to unbound drug concentration in the plasma against the bound drug concentration in the RBCs (C_R/C_{fP} vs. C_R). These data were obtained from samples of all 17 subjects treated with single 25 mg, 50 mg and 100 mg doses in this study. The unbound-plasma concentrations (C_{fP}) were 45% of the total plasma concentrations (Bayne et al., 1981). Using a Rosenthal plot (Rosenthal, 1967), Bayne et al. (1981) further estimated the concentrations of isoenzymes I and II to be 165 μM and 18 μM, respectively, and their dissociation constants were 0.6 μM and 0.007 μM, respectively.

The presence of two types of isoenzymes with unequal binding affinities for MTZ might explain the dose disproportionality observed in blood, plasma and RBCs in this study at drug concentrations below the K_m values. At low (25 mg and 50 mg) doses, MTZ binds quantitatively to type II isoenzyme (CA-II) in RBCs due to strong enzyme affinity, with little drug being available in plasma (Fig. 3A). At the 100 mg dose, all binding sites of CA-II become saturated, and the drug begins to bind to type I isoenzyme (CA-I). Because of the low affinity of CA-I, a portion of the drug becomes available to the plasma volume and concentrations in plasma rise accordingly (Fig. 3A). At a 300 mg dose, all CA-II and CA-I binding sites are perhaps occupied, and more drug distributes to the plasma, causing a marked increase in plasma drug concentrations (Fig. 3B). Therefore, when assessed in whole blood or RBCs, MTZ dose disproportionality appeared between the 150 mg and 300 mg doses. When assessed in plasma, practically no increase in drug concentration was observed between the 25 mg and 50 mg doses, and a sharp increase causing an apparent non-linearity appeared between the 50 mg and 100 mg doses. Plasma concentrations increased dose-proportionally from 100 mg to 150 mg doses (Fig. 3B). Above 150 mg doses, the increase of drug concentration in plasma becomes non-linear as in the case of whole blood or RBCs. Although the actual mechanism of action of MTZ is unknown at the present time and might be more complex, our proposal of preferential and sequential binding of MTZ to CA-II over CA-I is fully supported by the most recent findings on the

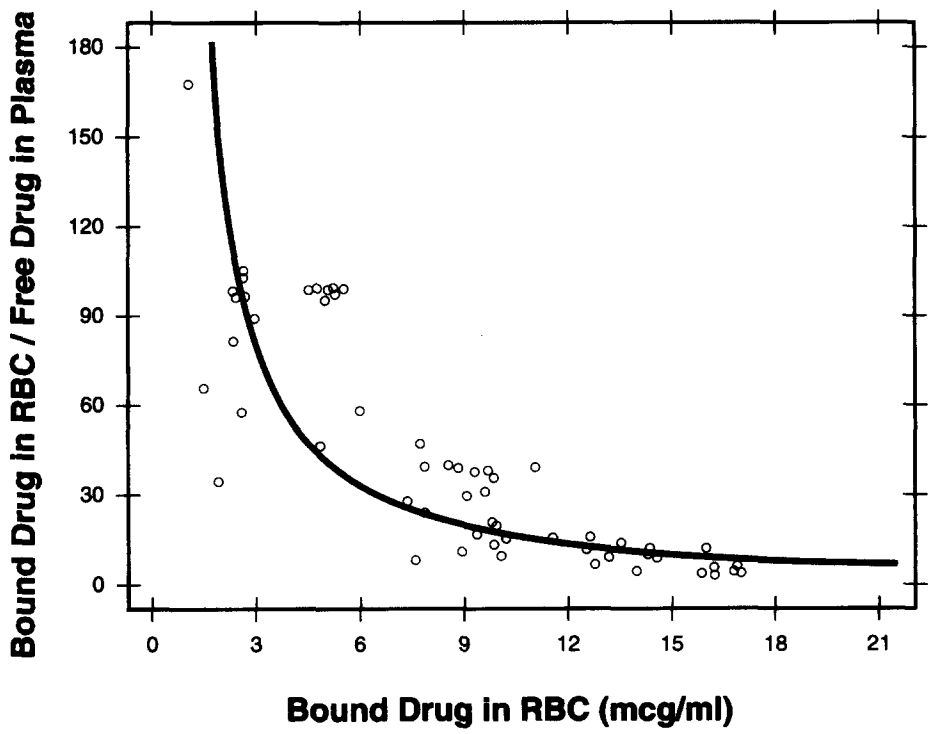

Fig. 4. Methazolamide binding curve obtained from data of samples of 17 subjects treated with single 25, 50, or 100 mg doses shows the relationship of the ratio C_R/C_{fP} against C_R, where C_R and C_{fP} are the bound drug concentration in RBCs and unbound drug concentration in plasma, respectively.

topically administered dorzolamide (Sugrue et al., 1993; Biollaz et al., 1995). As MTZ, dorzolamide is also a CA inhibitor used in the treatment of glaucoma. Recent finding indicated that dorzolamide is highly specific for human CA-II binding. It is about 3000 times more active against human CA-II than against human CA-I.

For the past several years both the Agency and pharmaceutical industries have been facing tremendous complexities in formulating guidelines for the bioequivalence study on MTZ drug product. From the in vivo studies (using HPLC assay procedures) with different doses of MTZ presented here, it is evident that MTZ concentrations in whole blood and in RBCs exhibit linear kinetics at least up to 150-mg doses, while the drug concentration in plasma is dose-proportional only from 100 mg to 150 mg. Plasma drug concentrations at 100-mg doses are too low to provide a reliable measure, and a dose higher than 100 mg may cause adverse reactions in healthy individuals (Physician's Desk Reference, 1995). Furthermore, the experimental procedure for determination of RBC drug concentration is very complex and time consuming. For these reasons, it is concluded that the pharmacokinetic parameters derived from whole blood analysis following a 100 mg dose is adequate for bioequivalence evaluation of multi-source MTZ formulations. Other analytical techniques, such as GLC-mass spectrometric methods may produce better results with somewhat higher accuracy. However, the availability of such equipment is limited.

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