The Potential Influence of CO₂, as an Agent for Euthanasia, on the Pharmacokinetics of Basic Compounds in Rodents

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ABSTRACT:

Rodent tissue distribution and pharmacokinetic studies were performed on basic compounds Org A and Org B in support of central nervous system drug discovery programs. A consistent observation from these studies was that drug concentrations in plasma obtained by cardiac puncture after CO₂ euthanasia were markedly higher compared with those from other sampling methods (serial sampling, isoflurane anesthesia, or cervical dislocation). Further investigations demonstrated that CO₂ euthanasia led to a reduction in blood pH in both rats and mice, which was not observed with the other sampling methods. The use of CO₂ euthanasia resulted in a decrease in the brain/plasma ratio of Org B, largely as a result of increased plasma concentrations. The pharmacokinetics of a basic drug, raloxifene, in rat were also influenced by sampling technique. CO₂ euthanasia before sampling, resulted in a 2- to 3-fold increase in the area under the drug concentration-time curve, a decrease in plasma clearance, and a decrease in the steady-state volume of distribution compared with isoflurane anesthesia. It is proposed that a decrease in the pH of blood relative to that of other tissues, as a consequence of CO₂ exposure, results in a redistribution of basic compounds out of the tissues, leading to higher concentrations in plasma.

For tissue distribution studies or combined tissue distribution/pharmacokinetic studies in rodents, a common approach is to administer the test compound to several groups of animals. Subsequently, at each designated time point, blood and tissue samples are taken from a group of animals after euthanasia. A survey of the literature indicates that various methods of euthanasia are used for these types of studies including CO₂ inhalation (Sauer et al., 1997; Doran et al., 2005; Becker and Liu, 2006; Venkatakrishnan et al., 2007), pentobarbital (Washington et al., 2000; Groll et al., 2005), isoflurane (Gustafson et al., 2006), halothane (Hepburn et al., 2001), and decapitation (Hong et al., 2006). In many cases the method of euthanasia was not reported.

At Organon, various central nervous system discovery programs have entailed the study of the pharmacokinetics and brain distribution of drug candidates in both mice and rats. Until recently, the typical sampling design of these studies in our laboratory had been as follows. For rat pharmacokinetic studies, serial blood samples were taken from a tail vein with a terminal sample taken by cardiac puncture (CP) after CO₂ euthanasia. For rat and mouse brain distribution studies and mouse pharmacokinetic studies, groups of animals were sacrificed at various time points (CO₂) before blood sampling (CP) and tissue harvesting.

Data from several unconnected studies in our laboratory suggested that, for some basic compounds, the different methods of sample collection (CP versus tail vein) resulted in differences in the observed drug concentration in plasma. Although the dependence of drug concentration on sampling site is well established (Chiou, 1989), this dependence could not adequately explain all of the observations. An alternative hypothesis was that the use of CO₂ before blood sampling resulted in an alteration in physiological pH giving rise to changes in compound distribution and hence changes in plasma concentrations. Further studies were performed with the aim of investigating this hypothesis. Data were generated for two proprietary small molecules (Org A and Org B) as well as raloxifene (Evista); all three compounds are basic with pKₐ values of 9.48, 9.69, and 7.33, respectively. The initial observations and the results of these follow-up studies will be described and discussed.

Materials and Methods

Materials. Raloxifene was obtained from Sigma-Aldrich (Poole, UK). Org A and Org B were synthesized at Organon Laboratories Ltd. (Newhouse, UK). Solvents and other reagents were obtained from common sources and were of reagent grade or better. For oral administration, Org A (1 mg/ml) was suspended in 0.5% (w/v) gelatin in 5% (v/v) mannitol. Org B (1 mg/ml) was dissolved in isotonic saline for both oral and i.v. administration. Raloxifene (1 mg/ml) was suspended in 5% (w/v) mulgofen in isotonic saline for oral administration and dissolved in 10% (v/v) dimethyl acetamide in water for i.v. administration.

Animals. Male Wistar BRL rats (200–300 g) and male mice (ICR or MF1, 20–30 g; Harlan, Bicester, UK) were maintained on a 12-h light/dark cycle in a temperature- and humidity-controlled room with access to water and food ad libitum. All procedures involving animals were governed by a Project license granted by the UK Home Office under the Animals (Scientific Procedures) Act 1986.

Plasma Pharmacokinetics. Animals were administered test compound either orally or i.v., and blood samples were collected at predetermined time points. Dose levels were as indicated in the appropriate tables and figures. For rat studies, blood samples were taken from either a lateral tail vein or from an arterial line.
indwelling jugular vein or carotid artery cannula. For mouse studies, blood samples were taken by CP.

**Multiple Sampling Site Studies.** Rats were administered test compound orally (Org A, 10 mg/kg; Org B, 5 mg/kg). At 4 h postdose, blood samples were obtained from a lateral tail vein and an indwelling carotid artery cannula. After euthanasia by overexposure to CO2, further blood samples were collected from the heart (CP), jugular vein, trunk, and thoracic artery.

**Termination Method Studies.** Three groups of mice (ICR, n = 8) were administered Org A (10 mg/kg) orally. At 1 h postdose blood (CP) and brain samples were collected from each group after euthanasia by either cervical dislocation (CD) or CO2 inhalation or after anesthesia by isoflurane.

**Blood Gas Analysis.** Rats or mice (ICR), were sacrificed by one of three methods: CD, CO2 inhalation, or isoflurane anesthesia followed by CD. A blood sample was obtained by CP from each animal and assayed using a blood gas analyzer (ABL500, Radiometer Ltd., Crawley, UK) to obtain blood pH and pCO2.

**Sample Preparation and Liquid Chromatography-MS/MS Analysis.** All blood samples were centrifuged (4000g for 10 min at 4°C) to obtain plasma. Brains were collected into ice-cold phosphate-buffered saline (pH 7.4), rinsed with a further aliquot of the same buffer, blotted dry on filter paper (Whatman, Maidstone, UK), and weighed. Plasma and brain samples were stored at −20°C before analysis by high-performance liquid chromatography-MS/MS. Samples were quantified using either and API-ESI-MS/MS (turbo ion spray source, 300°C; or an API-3000 (turbo ion spray source, 300°C; PerkinElmer-Sciex Instruments, Boston, MA) quadruple mass spectrometer. Plasma samples (50 µl) were prepared for analysis by protein precipitation using 150 µl of acetonitrile (MeCN) containing 100 ng/ml of an appropriate internal standard. The samples were vortex-mixed and centrifuged (4000g for 10 min at 4°C), and an aliquot of supernatant was removed for analysis. Before analysis, 100 µl of water was added to each supernatant sample. Brain samples were homogenized after addition of 3 volumes of phosphate-buffered saline. An aliquot (200 µl) of the homogenate was removed, and protein was precipitated by addition of 600 µl of MeCN containing the internal standard. The samples were vortex-mixed and centrifuged (4000g for 10 min at 4°C) and an aliquot of supernatant was removed for analysis. Before analysis, 250 µl of water was added to each supernatant sample, and each sample was analyzed in triplicate. Samples were injected (10–50 µl; CTC Analytics, Zwingen, Switzerland) onto a Phenomenex 50 × 2 mm, 5-µm Develosil C30 column (Phenomenex, Macclesfield, UK) at room temperature. The run time varied for each analyte (3.5–6 min). Raloxifene and Org A were eluted with a linear gradient consisting of 0.1% formic acid in water (A) and 0.1% formic acid in MeCN (B) produced by two binary pumps [raloxifene, Shimadzu LC-10AD VP (Shimadzu, Milton Keyes, UK); Org A, PerkinElmer series 200 micropumps (PerkinElmerSciex Instruments)]. Org B was eluted with a linear gradient consisting of ammonium formate in water (10 mM) (A) and methanol (B) produced by two Shimadzu LC-10ADVP binary pumps (Shimadzu). For Org A, the initial condition was 100% A held for 1 min then ramped to 100% B over 3 min and held for 0.5 min. The initial condition was returned to over 0.5 min and then held for a further 1 min. Org B conditions started at 90% A held for 1 min then ramped to 100% B over 0.5 min and held for 0.5 min and then returned to initial conditions over 0.5 min and held for a further 1 min. Raloxifene initial conditions were 100% A held for 1 min then ramped to 100% B over 1 min and held for 1 min. Initial conditions were returned to over 0.1 min and held for 0.9 min. For all analytes, the flow rate was 1 ml/min. Standards, quality control, and blank samples were prepared with either plasma or brain homogenate and were identical in composition to corresponding test samples. Accuracy of standards and quality control samples was within ±20% of the nominal value. The MS/MS transitions were 497.3/126.3 (Org A), 272.1/110.25 (Org B), and 474.16/112.25 for raloxifene.

**Data Analysis.** Data processing was performed using Excel (Microsoft, Redmond, WA) and WinNonlin v5.0.1 (Pharsight, Mountain View, CA). Statistical analyses were performed using Minitab release 14 (Minitab Inc., State College, PA).

**Results**

**Plasma Pharmacokinetic Studies.** Data from a brain penetration study and a pharmacokinetic study with Org A are shown in Fig. 1. Plasma samples obtained by CP after CO2 euthanasia gave consistently higher concentrations compared with those obtained by tail venipuncture. The estimated difference in the AUC was approximately 3-fold although, because of differences in the design of the two studies, no statistical comparison was performed. Data from a pharmacology study with Org A, in which the same rats were sampled by two methods, are given in Table 1. The plasma concentrations at 2 h after oral administration were between 2- and 3-fold greater in the CP samples compared with the jugular vein samples across a range of doses (3–30 mg/kg).

After i.v. administration of Org B to rats, the plasma concentrations from samples taken at 24 h from the carotid artery were 2- to 3-fold lower than those from samples taken immediately afterward (from the same animals) by CP after CO2 euthanasia (Fig. 2). After i.v. administration to male MF1 mice, plasma levels of raloxifene obtained after CO2 euthanasia were consistently higher than those obtained after isoflurane anesthesia (Fig. 3). Estimated noncompartmental pharmacokinetic parameters (sparse analysis) with the CO2 sampling method yielded a higher AUC, lower plasma clearance, and lower steady-state volume of distribution (Vss) compared with the isoflurane technique (Table 2).

**Multiple Sampling Site Studies.** Further studies with Org A and Org B in rats indicated that there was a difference in the plasma concentrations between samples obtained before and after CO2 euthanasia from a variety of sampling sites. The trend of the mean data, shown in Figs. 4 and 5 was also reflected in each individual animal (data not shown).

**Termination Method Studies.** The plasma and brain concentrations obtained 1 h after single oral administration of Org B to mice (10 mg/kg) are shown in Fig. 6. Plasma concentrations from mice sacrificed by CD were similar to those obtained under isoflurane anesthesia whereas the mice sacrificed by CO2 euthanasia showed substantially higher concentrations. The brain concentrations of Org A appeared broadly similar between the three groups. Brain/plasma ratios derived from these data were 52.8, 45.4, and 23.5 for the CD, isoflurane, and CO2 groups, respectively.

**Blood Gas Analysis Studies.** In both rat and mouse, the pH of blood obtained after CO2 euthanasia was markedly lower than that obtained by either CD or isoflurane (Table 3).

**Discussion**

The plasma concentration-time profile for Org A (Fig. 1) shows a clear difference between samples taken by CP after CO2 euthanasia and those taken from a tail vein. Similar observations were made in a
pharmacology study (Table 1) with the CP samples giving consistently higher concentrations of Org A than the jugular vein samples from the same animals. Sample site-dependent differences in drug levels have been discussed by Chiou (1989). For drugs that distribute into tissues, marked differences in concentration between arterial and venous samples have been observed. In the early (distributional) phase after drug administration, venous concentrations can be much lower because of the net uptake of drug during its passage across the tissues. During the elimination phase, with decreasing arterial concentrations, drug will redistribute out of the tissues, resulting in higher venous concentrations. Because the concentrations of Org A in the CP samples (Fig. 1) are consistently higher then the venous samples throughout the study, it is not clear that the phenomena discussed by Chiou (1989) could be responsible for the observations, although a more complete profile may have aided interpretation. Furthermore, from the imprecise nature of CP sampling, it is not possible to say whether this sample represents arterial blood, venous blood, or a mixture. Differences in plasma concentration between sampling methods were also observed during a study of the pharmacokinetics of Org B in rats (Fig. 2). Concentrations of Org B were appreciably higher in a CP sample (CO2 euthanasia) taken from the same animals immediately after a carotid artery sample. This observation is not inconsistent with sample site-dependent differences (Chiou, 1989).  

To investigate these initial observations further, the concentrations

### TABLE 1

<table>
<thead>
<tr>
<th>Dose</th>
<th>Plasma Concentration</th>
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<tbody>
<tr>
<td>ng/ml</td>
<td>CP After CO2 Euthanasia</td>
<td>Jugular Vein Cannula</td>
<td></td>
</tr>
<tr>
<td>3 mg/kg</td>
<td>202 ± 106**</td>
<td>66 ± 18</td>
<td></td>
</tr>
<tr>
<td>5 mg/kg</td>
<td>437 ± 151***</td>
<td>143 ± 53</td>
<td></td>
</tr>
<tr>
<td>10 mg/kg</td>
<td>866 ± 163***</td>
<td>437 ± 75</td>
<td></td>
</tr>
<tr>
<td>30 mg/kg</td>
<td>3346 ± 979***</td>
<td>1640 ± 335</td>
<td></td>
</tr>
</tbody>
</table>

Statistically different from jugular vein group by one-sample t-test: **P < 0.01, ***P < 0.001.

### TABLE 2

Noncompartmental pharmacokinetic parameters of raloxifene estimated after a single i.v. administration (1 mg/kg) to male MF1 mice

<table>
<thead>
<tr>
<th>Sampling Method</th>
<th>CO2</th>
<th>Isoflurane</th>
</tr>
</thead>
<tbody>
<tr>
<td>AUC0–7.5 h (ng · h/ml) (95% confidence intervals)</td>
<td>334 (317, 351)***</td>
<td>161 (147, 176)</td>
</tr>
<tr>
<td>Plasma clearance (ml/min/kg)</td>
<td>49</td>
<td>98</td>
</tr>
<tr>
<td>Vss (l/kg)</td>
<td>2.7</td>
<td>9.0</td>
</tr>
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***Statistically different from isoflurane, P < 0.001; z test after sparse analysis in WinNonlin.

Fig. 2. Plasma concentrations of Org B after a single i.v. administration to male Wistar BRL rats (3 mg/kg, mean ± S.D., n = 3). □, samples taken from the carotid artery; ●, a sample taken by CP after CO2 euthanasia. *, statistically different from carotid artery sample (p < 0.05 by one-sample t test).

Fig. 3. Plasma concentrations of raloxifene after a single i.v. administration to male MF1 mice (1 mg/kg, mean ± S.D., n = 4 per time point). ■, samples taken under isoflurane anesthesia; ●, samples taken after CO2 euthanasia.

Fig. 4. Plasma concentrations of Org A from various sampling sites at 4 h after a single oral administration to male Wistar BRL rats (10 mg/kg, mean ± S.D., n = 4). Samples were taken either before (shaded bars: TV, tail vein; CA, carotid artery) or after (open bars: CP, cardiac puncture; JV, jugular vein; TR, trunk; TA, thoracic artery) CO2 euthanasia. *, statistically different from CA (p < 0.05, one-sample t test).

Fig. 5. Plasma concentrations of Org B from various sampling sites at 4 h after a single oral administration to male Wistar BRL rats (5 mg/kg, mean ± S.D., n = 4). Samples were taken either before (shaded bars: TV, tail vein; CA, carotid artery) or after (open bars: CP, cardiac puncture; JV, jugular vein; TR, trunk; TA, thoracic artery) CO2 euthanasia. *, statistically different from CA (p < 0.05, one-sample t test).
of Org A and Org B from various sampling sites were studied after oral administration to rats (Figs. 4 and 5). For both compounds, concentrations from various sampling sites (both arterial and venous) taken after CO₂ euthanasia were higher than those taken before CO₂ euthanasia. It was thought that the carotid artery and thoracic artery samples would not be susceptible to sample site differences and that a statistical comparison would therefore be justified. The thoracic artery concentrations (after CO₂) were significantly higher than the carotid artery samples (before CO₂) for both Org A (4.7-fold) and Org B (3.3-fold). This finding suggested that other factors aside from sample site and arteriovenous differences were responsible for the observations.

Lipophilic basic drugs are characterized by high volumes of distribution as a consequence of extensive uptake into tissues. The mechanisms governing tissue uptake are thought to be an interplay between phospholipid binding and ion trapping (Römer and Bickel, 1979; MacIntyre and Cutler, 1988; Hung et al., 2002; Siebert et al., 2004). Ion trapping occurs as a consequence of the pH differences between blood, tissues, and subcellular organelles, especially lysosomes, which are maintained at a pH range of 4 to 5 (de Duve et al., 1974). For basic drugs, a greater proportion of the drug will exist in the protonated form in lower pH environments. Because the protonated form is considered to be unable (or only sparingly able) to cross biological membranes, the net result is a sequestration of drug into compartments of lower pH. Clearly then, a change in the pH of blood, tissues, or lysosomes has the potential to alter the distribution characteristics of an ionizable drug as has been reported in several laboratories (Lüllmann et al., 1985; Shibasaki et al., 1989; Ishizaki et al., 1998). Indeed, modulation of distribution via alterations in pH has been proposed as a means of enhancing the efficacy of anticancer drugs (reviewed by Kaufman and Krise, 2007).

In the current study, euthanasia of rats and mice with CO₂ resulted in markedly lower blood pH (Table 3) as a consequence of an increase in blood pCO₂ (data not shown). Changes in arterial blood and tissue intracellular pH in rats, in response to CO₂ inhalation, has previously been reported by Rothe (1984). In arterial blood, the fall in pH was approximately twice that observed in liver, spleen, and heart muscle, whereas skeletal muscle pH appeared resistant to mild acidosis. In contrast, changes in brain pH were quantitatively similar to those in arterial blood (Rothe, 1984). In the current study, anesthesia with isoflurane did not appear to affect blood pH compared with the CD group. Although isoflurane has been reported to produce acidosis in mice (Sjöblom and Nylander, 2007), this was relatively mild (blood pH 7.15) and took 2 h to develop. Therefore, a possible explanation for the higher plasma levels of Org A and Org B after CO₂ euthanasia is that the fall in blood pH, relative to that in tissues, results in redistribution of compound out of the tissues and acidic organelles into the blood. In a brain distribution study with Org A in mice (Fig. 6), plasma levels were higher in the CO₂ euthanasia group compared with the isoflurane and CD groups. Brain tissue levels of Org A were slightly lower in the CO₂ group although this difference was not statistically significant. As a consequence mainly of the increased plasma concentration, the brain/plasma ratio of Org A derived from the CO₂ group (23.5) was approximately 2-fold lower compared with the isoflurane (45.4) and cervical dislocation (52.8) groups. It is possible that a relatively small redistribution from multiple tissues could lead to a marked increase in plasma concentrations. Whereas brain/plasma ratios give a useful indication of the distributional properties of a drug, cerebrospinal fluid drug levels are the preferred means, in our laboratory, of assessing central nervous system exposure with respect to pharmacological effect (Martin, 2004) and extrapolation to man. It would therefore be of interest to study the influence of CO₂ on drug levels in cerebrospinal fluid in relation to plasma.

A study of the pharmacokinetics of raloxifene in mice after i.v. administration provided further evidence of the influence of CO₂. The plasma levels of raloxifene obtained after CO₂ euthanasia were consistently higher than those obtained under isoflurane anesthesia (Fig. 3) and yielded a 2-fold higher AUC (p < 0.001). This difference was reflected in the estimated plasma clearance and V₅₀ (Table 2). Again, it is suggested that exposure to CO₂ immediately before sampling and the concomitant fall in blood pH resulted in a redistribution of this basic compound out of the tissues, leading to higher observed plasma levels.

A series of observations from unconnected studies and subsequent follow-up investigations have provided evidence that different methods of blood sample collection can lead to differences in the data obtained. The results of these investigations led us to suspect that the use of CO₂ euthanasia in tissue distribution and pharmacokinetic studies on basic compounds could yield potentially artifactual plasma concentration data. We are aware of several literature reports in which CO₂ euthanasia has also been used during tissue distribution or pharmacokinetic studies on other basic compounds (Handal et al., 2002; Debruyne et al., 2003; Polli et al., 2003; Meririnne et al., 2004;...
Doran et al., 2005; Liu et al., 2006; Venkatakrishnan et al., 2007). In one of these reports (Meririnne et al., 2004), rat tissue samples were obtained after decapitation under light sedation with CO₂. It was stated that preliminary tests demonstrated that tissue concentrations (including blood and brain) of 4-methylaminorex were not affected by light exposure to CO₂. Because these data were not reported it is difficult to make comparisons with data obtained in the current study (Fig. 6) and assess the possible difference between light sedation and euthanasia. Nonetheless, the results presented here suggest that a reevaluation of data generated from studies using CO₂ anesthesia or euthanasia may be warranted. Further studies would be required to clarify the mechanisms responsible for the observations described in this article including investigating the effect of CO₂ on pH and drug distribution in various tissues. Studies on compounds with a wider range of basic pKₐ values and lipophilicities as well as acidic compounds are also indicated. Although not thought to be relevant to the compounds in the present study, the potential influence of CO₂ on reversible metabolism should be considered in future work of this type. The data presented here have prompted a change to isoflurane sedation.

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References


Gustafson DL, BRADSHAW-PIERCE EL, MERTZ AL, and ZIRROLLI JA (2006) Tissue distribution and range of basic pKₐ distribution in various tissues. Studies on compounds with a wider range of basic pKₐ values and lipophilicities as well as acidic compounds are also indicated. Although not thought to be relevant to the compounds in the present study, the potential influence of CO₂ on reversible metabolism should be considered in future work of this type. The data presented here have prompted a change to isoflurane anesthesia in our laboratory.

Influence of CO₂ on Rodent PK


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