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# Communications to the Editor

## Simultaneous Pharmacokinetic Screening of a Mixture of Compounds in the Dog Using API LC/MS/MS Analysis for Increased Throughput

Judd Berman,* Kathy Halm, Kim Adkison, and Joel Shaffer

*Glaxo Wellcome, Five Moore Drive, Research Triangle Park, North Carolina 27709*

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Drug discovery increasingly relies on the ability to rapidly identify "quality" molecules that possess the desired attributes of bioavailability, chemical tractability, selectivity, and potency. Traditional methods used to determine the pharmacokinetics (oral bioavailability, clearance, volume of distribution, and half-life) of molecules have presented a bottleneck in some drug discovery programs. We have increased throughput in in vivo pharmacokinetic screening of structurally related compounds by dosing mixtures of compounds intravenously to a single animal and using atmospheric pressure ionization (API) tandem liquid chromatography/mass spectrometry (LC/MS/MS) for analysis. We have referred to this as *N*-in-one dosing where *N* is the number of compounds coadministered. The method was used to simultaneously determine the clearance (CL), steady-state volume of distribution ($V_{ss}$), and elimination half-life ($t_{1/2}$) of five $\alpha_{1a}$ receptor antagonists (compounds possessing selective $\alpha_{1a}$ antagonist properties may have potential therapeutic importance in the treatment of benign prostatic hyperplasia).[1−3] The mixture approach provides an opportunity to study the pharmacokinetics of several compounds under identical conditions while minimizing sample processing time and the number of animals required. To the best of our abilities, we found limited literature that capitialized on the advantages gained through simultaneously dosing compounds to determine pharmacokinetics and bioavailability after intravenous and intraduodenal administration,[4,5] and one of these utilized LC/MS.[6]

Five $\alpha_{1a}$ receptor antagonists (Figure 1) that had been previously studied by individual dosing (known range of CL, $V_{ss}$, and $t_{1/2}$) were dosed intravenously as a mixture to a single dog. In the traditional individual dosing studies, plasma samples were analyzed for $\alpha_{1a}$ antagonist by reverse-phase HPLC with fluorescence detection. Plasma sample analysis of the $\alpha_{1a}$ antagonists in the mixture study relied heavily on the application of atmospheric pressure ionization (API) LC/MS methodology using a triple quadrupole instrument. LC/MS with its inherent detection specificity, selectivity, and sensitivity enabled rapid analytical method development prior to dosing the animal as well as high throughput sample analysis.

The compounds studied exhibited good mass spectrometric response in the positive ion mode using the API technique. To increase analyte specificity from the biological matrix, a reverse-phase LC/MS/MS method was developed. For this series of compounds, a characteristic neutral loss of $CF_3CH_2OH$ (100 amu) was observed in the product ion mass spectra. This transition was optimized for sensitivity in the selected reaction monitoring (SRM) mode. Among the compounds studied, a pair of isobars (compounds **2** and **3** of molecular weight 638) produced the same abundant fragment ion at *m*/*z* 539. Consequently, the HPLC mobile phase conditions were optimized to resolve compounds **2** and **3**, extending the analysis time to 6 min. An internal standard (compound **6**) was added to correct for possible changes in sample extraction or instrument performance during the analysis. Figure 2 shows LC/MS/MS SRM reconstructed ion chromatograms obtained from a 50 ng/mL plasma calibration standard. Compound **3** coeluted with the internal standard (compound **6**), while the isobaric pair, compounds **2** and **3**, were chromatographically resolved. Quantitation was carried out over the range of 6−2500 ng/mL (seven points). Concentration data were calculated using the ratio of the peak area of each compound to the peak area of the internal standard. Linear regression analysis with $1/x$ weighting was used to fit all of the calibration standard curves, and yielded

* Address correspondence to: Judd Berman, Glaxo Wellcome, Five Moore Dr, Research Triangle Park, NC 27709. Tel: (919) 483-1174. FAX: (919) 315-0430. E-mail: berman~j@glaxo.com.

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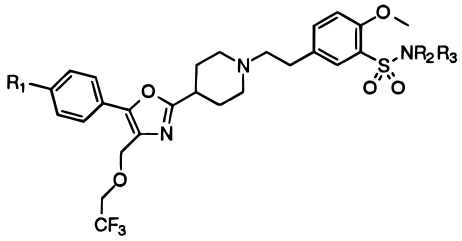

**1**, $R_1$=H; $R_2$=H; $R_3$=H
**2**, $R_1$=H; $R_2$=$CH_3$; $R_3$=$CH_2CONHCH_3$
**3**, $R_1$=H; $R_2$=H; $R_3$=$CH_2CH_2NHCOCH_3$
**4**, $R_1$=F; $R_2$=$CH_3$; $R_3$=$CH_2CONHCH_3$
**5**, $R_1$=F; $R_2$=H; $R_3$=H
**6**, $R_1$=H; $R_2$=H; $R_3$=$CH_2CONH_2$

**Figure 1.** Structures of compounds **1**−**6**.[8]

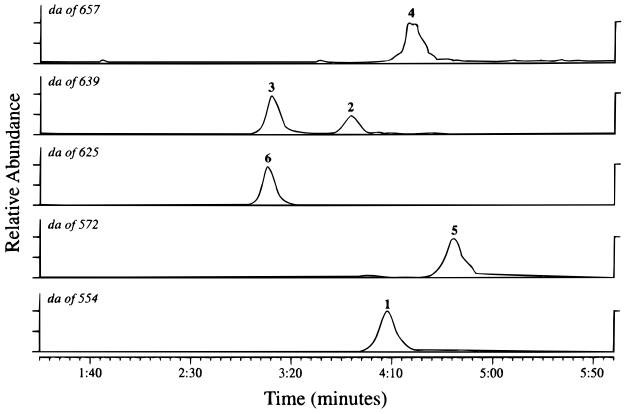


**Figure 2.** API LC/MS/MS reconstructed ion chromatograms obtained from a 50 ng/mL plasma calibration standard. Compounds numbered as in text.

correlation coefficients of greater than 0.99. The intercepts of the lines were not significantly different from zero, and the percent difference from the line for the lowest concentration calibration standards was within 26% for each of the compounds.

The concentration−time profiles of the compounds from the mixture study are shown in Figure 3. The concentration−time profiles were biphasic, and the shapes were similar to those observed for each compound after individual dosing (data not shown). The pharmacokinetic parameters of the compounds from the mixture study and from individual dosing are given in Table 1. There was a good correlation (slope = 1.08; $r^2$ = 0.90) in $t_{1/2}$ of the five compounds between the mixture and individual studies (Figure 4). In general, the CL ($r^2$ = 0.55) and $V_{ss}$ ($r^2$ = 0.50) of the compounds also compared favorably between the mixture and individual studies (with the exception of compound **3**). The $V_{ss}$ of compound **3** was 4-fold higher after mixture dosing compared to individual dosing and the CL of compound **3** was 2-fold greater. The elimination $t_{1/2}$ is proportional to the volume of distribution and inversely proportional to the CL; thus the parallel increase of CL and volume resulted in only a modest change in the observed $t_{1/2}$ of compound **3**. It should be noted that individual determinations of the pharmacokinetic parameters of compounds **2**−**5** were conducted in different dogs. Some of the differences between individual and mixture dosing may be related to interdog variability. Additionally, differences between individual and cassette dosing could be attributed to plasma protein binding displacement or inhibition of metabolism. The data presented here are insufficient to ascertain which of these possibilities is most likely.

In vivo pharmacokinetic SAR shows that substitution of $R_2$ and $R_3$ (Figure 1) leads to increased $V_{ss}$ and increased CL (compare compounds **2**, **3**, and **4** with compounds **1** and **5**). Substitution of F for H at $R_1$ results in decreased CL (compare compounds **4** and **2** and especially compounds **5** and **1**). The decrease in CL most likely results from decreased metabolism since renal excretion of parent accounts for less than 2% of the CL of these compounds (data not shown). Compound **5** has a low CL (less than one-tenth liver blood flow) and a $V_{ss}$ suggestive of an attractive pharmacokinetic profile for a systemically active agent.

The perceived major advance of the mixture approach described relates to the dramatic increase in the numbers of compounds that can be studied simultaneously and the ability to generate in vivo pharmacokinetic SAR. Nonetheless, there are also *potential limitations* with the mixture approach *that merit further investigation and caution*. For example, there may be molecule−molecule interactions that alter metabolism, distribution (either plasma or tissue protein binding), or renal/biliary excretion. We did not see any evidence for inhibition of metabolism or plasma protein displacement. Inhibition was most likely avoided due to the low plasma concentrations achieved from the doses administered. Generally, plasma concentrations in the nanomolar range are not high enough to inhibit enzymes involved in xenobiotic metabolism or to cause plasma protein binding displacement.[7] Complications may arise in mixture studies due to pharmacologic or toxicologic events that limit the total dose or number of molecules that can be coadministered. We observed no adverse effects from coadministration of these adrenergic blocking agents. Additionally, whereas mass spectrometry provided very sensitive and selective detection for the analysis of multiple compounds in plasma, it is important to recognize that several analytical complications could arise from redundancy in molecular weight or HPLC retention characteristics. For example, specificity issues where metabolites coeluted with analytes of identical mass and fragmentation monitored in the SRM mode would lead to inaccurate plasma concentrations. The compounds in this study had similar chromatographic characteristics, and isocratic HPLC was used for the separation. Mixtures of more diverse compounds may require gradient elution HPLC and consequently extend the analysis time. There may also be solubility considerations in formulating a dosing vehicle containing more than one compound. Some of these complications may be alleviated by taking these factors into account in the selection of compounds for coadministration. The studies reported here were conducted in the dog. The use of other species (e.g.; rat, mouse) may present further challenges and opportunities.

Dosing compound mixtures followed by API LC/MS/MS analysis offers a rapid in vivo screening aid in lead identification and lead optimization; this may help prioritize further more definitive studies. The greatest potential utility of the mixture approach may be to screen larger numbers of compounds and begin to develop databases of detailed in vivo pharmacokinetic SAR. This will lead to testable hypotheses about the role of various structural attributes in modifying ab-

**Table 1.** Pharmacokinetic Parameters of Five Compounds Obtained following Five-in-One and Individual Dosing in the Dog

| | half-life (h) | | clearance ((mL/min)/kg) | | $V_{ss}$ (mL/kg) | |
|---|---|---|---|---|---|---|
| compd | five-in-one | individual | five-in-one | individual | five-in-one | individual |
| **1** | 1.5 | 1.4 | 5.9 | 5.0 | 473 | 382 |
| **2** | 2.4 | 2.1 | 17.3 | 28.9 | 2757 | 3464 |
| **3** | 2.9 | 2.6 | 18.7 | 10.3 | 2833 | 600 |
| **4** | 3.0 | 3.4 | 11.2 | 11.6 | 2857 | 2907 |
| **5** | 4.9 | 4.2[a] | 0.86 | 0.90[a] | 330 | 243[a] |

[a] The parameters for compound **5** are the mean of $n = 3$ dogs. The other compounds were tested individually in one dog.

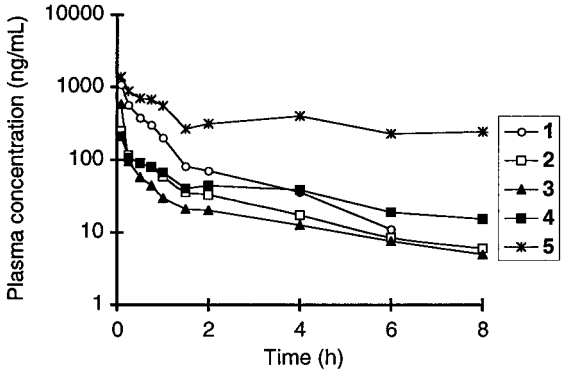


**Figure 3.** Concentration−time profiles of five $\alpha_{1a}$ antagonists following intravenous coadminstration in a dog. Only compound **5** was detectable (24 ng/mL) at 24 h.

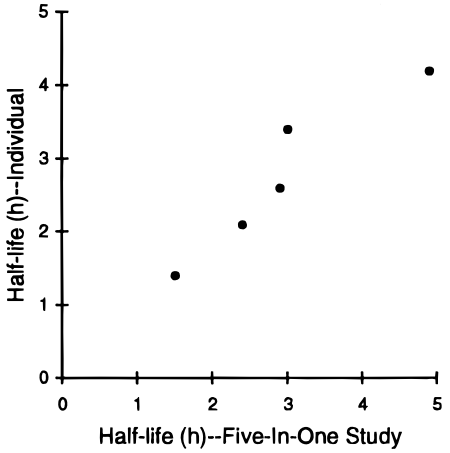


**Figure 4.** Comparison of the half-lives of five $\alpha_{1a}$ antagonists following individual dosing or five-in-one dosing.

sorption, distribution, metabolism, and excretion in future drug discovery programs. In addition, the use of this methodology may have special application in the planning and testing of combinatorial libraries. The number of compounds that can be tested simultaneously is dependent on the extent of drug−drug interactions, pharmacodynamic and toxicologic effects, and solubility of the mixture. *Studies that have extended the methodology to greater numbers of compounds per animal, as well as different structural classes of molecules, have been conducted* and will be reported in due course.

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**Supporting Information Available:** Experimental details (3 pages). Ordering information is given on any current masthead page.

**Note Added in Proof.** See: Olah, T. V.; McLoughlin, D. A.; Gilbert, J. D. The simultaneous determination of mixtures of drug candidates by liquid chromatography atmospheric pressure chemical ionization mass spectrometry as an *in vivo* drug screening procedure. *Rapid Commun. Mass Spectrom.* **1997**, *11*, 17−23.

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