Drug Pharmacokinetics Determined by Real-Time Analysis of Mouse Breath

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Drug pharmacokinetics determined by real-time analysis of mouse breath

Xue Li[a, d]‡, Pablo Martinez-Lozano Sinues[a]‡, Robert Dallmann[b, e]‡, Lukas Bregy[a], Maija Hollmén[a], Steven Proulx[a], Steven A. Brown[b], Michael Detmar[a], Malcolm Kohler[c], and Renato Zenobi*[a]

Abstract: Noninvasive, real-time pharmacokinetic (PK) monitoring of ketamine, propofol, and valproic acid, and their metabolites was achieved in mice, using secondary electrospray ionization and high-resolution mass spectrometry. The PK profile of a drug influences its efficacy and toxicity because it determines exposure time and levels. The antidepressant and anaesthetic, ketamine (Ket), and four Ket metabolites were studied in detail and their PK was simultaneously determined following application of different sub-anaesthetic doses of Ket. Oral bioavailability vs. intraperitoneal injection was also investigated. As opposed to conventional studies that require many animals to be sacrificed even for low-resolution PK curves, this novel approach yields real-time PK curves with a hitherto unmatched time resolution (10 s), and none of the animals has to be sacrificed. This thus represents a major step forward not only in animal welfare, but also major cost and time savings.

Current drug discovery programs rely heavily on the characterization of compounds with unknown *in-vivo* properties using animal models prior to the clinical phases of development.[1] During lead optimization, pharmacokinetic (PK) parameters of each compound are determined in plasma and possibly specific target organs of mice and rats. By determining the concentration of each compound and its degradation products over time in a given tissue, it is possible to estimate exposure levels after administration of a given dosage. However, since each time point in a PK profile typically requires the sacrifice of several animals, large amounts of compound have to be synthesized and potentially significant inter-individual variation is encountered. Therefore, it would be highly desirable to achieve such time-resolved analyses in individual animals. Aided by the development of highly sensitive bioanalytical methods, dried blood spot and microsampling techniques have been introduced recently to overcome some of these limitations[2]. Especially in mice this significantly reduces the number of animals and compound needed, and prevents inter-individual variation. Micro-sampling of blood at the tail, however, has the drawback of stressing the animal, which may change drug distribution and metabolism[3]. Although this problem can be overcome by using, for example, more invasive implanted catheters for automated sampling systems[4], the solution is far from ideal. Despite advances in high-throughput analyses by chromatographic and mass spectrometric methods, substantial time and effort are needed for sample preparation and analysis. Current methods thus still require significant resources, and are far from providing an instantaneous response to decide whether a candidate drug fulfills the requirements for further consideration.

Mouse breath could provide a matrix to overcome these limitations. Monitoring breath has received significant attention since the 1970s due to its non-invasive character[5]. Compared to plasma, blood, and other biosamples, analysis of breath is advantageous because: (i) the sampling is minimally- or non-invasive; (ii) there is no or little restriction on sampling volume and frequency; (iii) the matrix is relatively simple and clean. Non-invasive, real-time, on-line and direct analyses can therefore be readily achieved in breath[6].

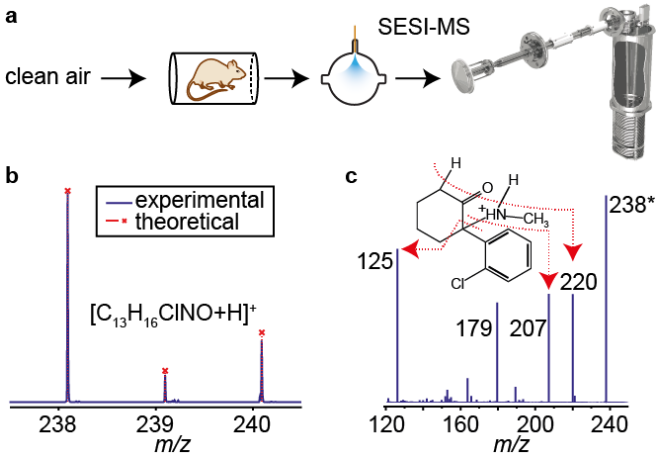

Figure 1. (a) Schematic of the experimental set-up. For details, see **Figure S1**. (b) SESI mass spectrum recorded from mouse breath, showing the isotope pattern in the m/z region of protonated Ket. (c) Fragment ion (SESI MS/MS) spectrum produced using m/z = 238 as precursor ion.

Here we report that secondary electrospray ionization high-resolution mass spectrometry (SESI-HRMS)[7] can be used to determine the PK profile of drugs non-invasively and in real time. Due to its sensitivity and its capability of detecting higher-mass drugs and their metabolites, SESI has important advantages over proton transfer reaction MS, which can monitor endogenous breath compounds in rats and mice.[8] In this study, we investigated the pharmacokinetics of different drugs, including ketamine (Ket) and its metabolites in the breath of male C57Bl/6J mice. Ketamine, an NMDA receptor antagonist, has been developed as a general anesthetic but is mostly used as an analgesic for severe pain and lately has shown some promise in

[a] Dr. X. Li, Dr. P. Martinez-Lozano Sinues, L. Bregy, Dr. M. Hollmén, Dr. S. Proulx, Prof. M. Detmar, Prof. R. Zenobi
Department of Chemistry and Applied Biosciences
ETH Zürich, CH-8093 Zürich, Switzerland
E-mail: Zenobi@org.chem.ethz.ch
[b] Dr. R. Dallmann, Prof. S.A. Brown
Institute of Pharmacology and Toxicology
University of Zürich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland.
[c] Prof. M. Kohler
Pulmonary Division
University Hospital Zürich, Rämistrasse 100, CH-8091 Zürich, Switzerland
[d] Dr. X. Li
Institute of Atmospheric Environmental Safety and Pollution Control
Jinan University Huangpu Road West 601, Guangzhou 510632, China
[e] Prof. R. Dallmann, University of Warwick, Warwick Medical School, Gibbet Hill Road, Coventry, CV4 AL7, United Kingdom
‡ These authors contributed equally to this work

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the treatment of depression[9]. After administering the drug, animals were placed in an acrylic chamber flushed with a constant flow of air, which was then directly analyzed by SESI-HRMS (Figure 1a, Figure S1 and Experimental Section).

To verify that Ket injection did not influence the drug measurements through mediating changes in respiration, we used whole body plethysmography. We found no significant difference to reported values of minute volume for B6 mice (~70 ml/min)[10], and Ket injection had no significant impact on the minute volume (data not shown).

Shortly after Ket administration to mice, we observed the increase of a signal cluster in the range *m/z* 238-240, which perfectly matched the isotopic distribution of the protonated molecule ($[M+H]^+$) of Ket (Figure 1b). Furthermore, fragment ions formed from the $[M+H]^+$ ion of Ket (Figure 1c) matched those previously reported[11], and fragments obtained from Ket standard (Figure S2a). Not only Ket, but also its main metabolites could all be detected with our set-up. Norketamine (NK)[11, 12] was unambiguously identified in mouse breath (Figures S2b and S3a). Three further metabolites of Ket were simultaneously detected (Figures S3 and S4): hydroxyketamine (HK), hydroxynorketamine (HNK) and dehydronorketamine (DHNK)[13].

Administration of multiple sub-anaesthetic doses of Ket led to concentration distributions similar to those previously reported, with a half-life ($t_{1/2}$) of 23 min (Figures 2a and S5), which agrees well with values obtained from plasma[14]. Note that a precise comparison between breath and plasma values would require very frequent plasma sampling, which is difficult or impossible with current methodologies due to volumetric constraints.

To estimate the biological variability of our method, we tested an additional batch of three mice injected with 45 mg/kg (Figure S6**)**. Variation of the maximum Ket signal intensity (I_{max}) was ~18 %. Compared to an increase in dose from 45 to 60 mg/kg, which resulted in an 86 % increase in I_{max} (i.e. from 2.4×10^5 to 4.4×10^5 counts in Figure 2a), this demonstrates that our method gives a robust and reproducible reading of drug levels.

We found an almost linear correlation of I_{max} and the injected drug concentration (Figure 2b), which is also consistent with the plasma behavior reported for Ket[15]. However, I_{max} was found to be delayed when compared to plasma values obtained from the literature[14]. This phenomenon could be attributed to the partitioning of Ket (and also its metabolites) between the blood and breath[16]. Compared to earlier studies, breath profiles of Ket and its metabolites coincide more closely with those expected for organ levels than for plasma; for example, Ket peaks 10-20 min in the liver, skin and muscle after i.v. injection in rats[17]. Thus, it is likely that breath analysis reports lung levels rather than plasma levels. Since most drugs target organs rather than blood itself, this kinetic difference could be significant and useful, and represents another potential advantage of breath analysis.

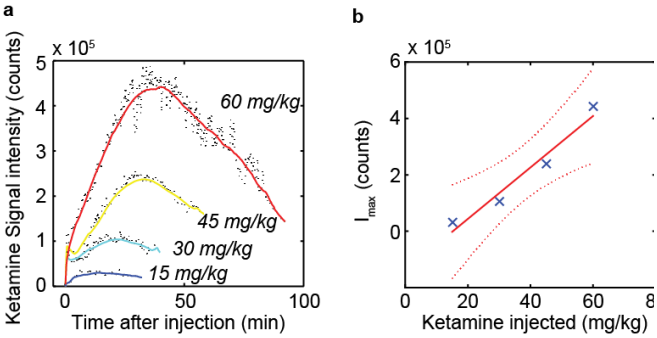

Figure 2. (a) Time-dependent Ket signal for four Ket doses, 15, 30, 45, and 60 mg/kg. Each dose was injected in a different mouse (n=4). Black dots represent the raw data and solid curves are smoothed data. (b) Dependence of the maximum Ket signal on the amount injected (r^2= 0.9563; p-value = 0.0221). Linear fit and confidence bounds are shown in red.

Similarly to Ket, its four metabolites showed a linear dose-response trend for I_{max} (Figure S7). As expected, the primary metabolites (i.e., NK and HK) peaked after Ket, which is consistent with results reported for NK in rat serum samples [13]. Although NK and HK have the same t_{max}, they showed a similar decay at 15 mg/kg, but very different decay at increasing dose (i.e. NK dropped at higher rate than HK; Figure S8).

Secondary NK metabolites (i.e., HNK and DHNK) peaked well after NK. Overall, it was consistently found at different doses that t_{max} (Ket) < t_{max} (NK) ≈ t_{max} (HK) < t_{max} (HNK) ≈ t_{max} (DHHK)(Figures 3a, S7 and S9**)** , while I_{max} (HK) < I_{max} (DHNK) < I_{max} (HNK) < I_{max} (NK) < I_{max} (Ket) (Figures 3b).

To study the relationship between the known plasma PK and our novel matrix, we compared the intensity of Ket and NK recorded in breath with the plasma concentrations from the same animal at that moment in time by terminal blood sampling (Figure 3c). A significant correlation was observed between breath and plasma levels for Ket (r^2=0.9498, p-value=0.0254) and NK (r^2=0.9739, p-value=0.0131). The concentrations in plasma depended on when the animals were sacrificed after Ket administration. The data in Figure 3c were obtained 32, 40 and 58 minutes after Ket administration of 15, 30 and 45 mg/kg, respectively. At this time, Ket had already started to be metabolized and redistributed to different body compartments, which explains the higher concentration of NK in plasma compared to Ket. In contrast, the peak intensity of NK in exhalation was only around one fifth of Ket (Figure 3b). Likely, this reflects the partitioning of Ket between blood and breath[16]. It may well be that breath levels reflect organ concentrations: the transport of NK from blood into the lung and breath should be less efficient than that of Ket due to the lower lipophicility of NK. This has been found for the blood-to-brain transport efficiency of NK, which is inferior to that of Ket[13]. However, further direct comparisons with LC/MS experiments on tissues would be needed to confirm this hypothesis. In addition, while translating ion intensity into absolute gas-phase concentrations is possible[18], it is instrument and compound-dependent. In this study, we report

on ion intensity, rather than absolute gas-phase concentrations.

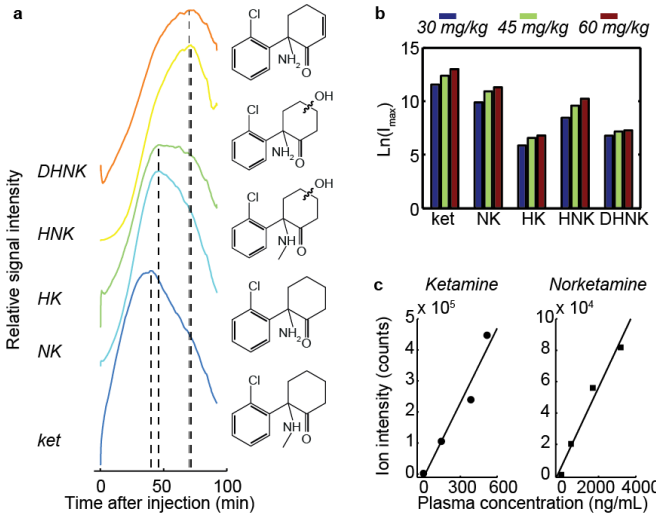

Figure 3. (a) Normalized signal intensity for Ket and its metabolites detected in the exhalation of a mouse injected with 60 mg/kg of Ket. Similar results have also been obtained for the doses of 45 mg/kg, 30 mg/kg and 15 mg/kg. The data shown are smoothed curves. The raw data can be found Fig. 2a (Ket) and Figure S7 (metabolites). Note the different kinetics. The times of the peak concentrations are: Ket = 34.5; NK = 46; HK = 51; HNK and DHNK = 77 min. (b) Maximum signal intensities of Ket and its metabolites, or 3 different doses (15, 30 and 45 mg/kg) of Ket and one negative control. (c) Exhalation intensity vs. blood concentration for Ket and NK.

In order to strengthen the potential of this technique for *in-vivo* real-time drug studies, we measured PK curves for additional drugs: propofol (widely used anesthetic) and valproic acid (antiepileptic drug). Figure S10 shows the isotopic distribution of propofol along with its fragmentation spectrum. The PK curve is shown in Figure S11, which is consistent with the literature[19]. Similarly, the PK curve of valproic acid and one of its metabolites (Figure S12) was found to be consistent with reported values[20].

Moreover, we further explored the possibility to rapidly estimate not only drug PK, but also bioavailability after oral dosing, because this is strongly preferred in most indications. Typically, this is tested early during drug discovery when only precious little compound is available. Therefore, we tested if we could use our method to estimate oral bioavailability and compared oral dosing of ketamine to IP dosing (Figure S13). We found an I_{max} for the oral dose of around 15% of that of the IP administration. This is consistent with literature in rats and humans[21].

In conclusion, we show SESI-HRMS analysis of mouse breath to be a promising strategy for minimally invasive, real-time pharmacokinetic monitoring using Ket, propofol, valproic acid, and their metabolites as examples. Perhaps the most significant advantage of the presented breath analysis method is that pharmacokinetic data based on mass spectrometric monitoring of exhalation is available instantaneously in real-time and superior time resolution from a single individual. Thus, our methodology not only results in more precise data with considerable time and cost savings, but also represents an important step forward in animal welfare, since multiple compounds can easily be measured repeatedly in a single animal. The fact that the proposed method enables obtaining a complete PK curve with one single dose/animal, may be advantageous when small quantities of compound are available in preclinical studies. Furthermore, drug/drug-interaction studies can be easily monitored, even if the compounds have completely different dosing schedules and kinetics. As an important secondary advantage, we hypothesize that pharmacokinetic profiles are obtained that reflect tissue rather than plasma timing.

We are confident that the SESI-HRMS method will be useful in pre-clinical programs and may even have potential applications in the clinical phases of drug development[7].

Experimental Section

Animals. All research involving animals had been approved by the Cantonal authorities of Zurich and are in accordance with the Declaration of Helsinki. Ketamine hydrochloride (Ketalar, Pfizer) was intraperitoneally (IP) injected into 3-month old male C57Bl/6J mice at 0, 15, 30, 45 and 60 mg/kg in a total volume of 100 µl phosphate buffered saline. Oral ket doses were administered at 45 and 135 mg/kg to two different mice (**Fig. S11**). One mouse at each of the IP and oral doses was used except for 45mg/kg, where 3 mice were injected. Propofol and valproic acid were IP injected at 25 and 18 mg/kg, respectively.

Plethysmography. Breathing was recorded using the constant flow-through whole-body plethysmography technique. Mice were placed in individual calibrated plethysmograph chambers (200 mL; EMKA Technologies, Paris, France) supplied with a constant airflow (600 ml/min). The animals were left to adapt in the plethysmograph chambers for approximately 30 min. Once they displayed a resting breathing rate, they were injected with ketamine or vehicle, and then replaced in their respective plethysmograph for an additional 60 min. of recording.

Breath sampling. After injection of Ket, mice were put in a plexiglass tube capped with O-ring sealed teflon stoppers. Mouse exhalation was delivered into a homemade SESI source (Figure S1b)[6] by flushing the chamber with 2 L/min compressed „medical air.“ The ESI nano-spray was formed from 0.2% aqueous formic acid, which was highly efficient for ionizing amines in positive ion mode[22] and acids in negative ion mode[23]. Ketamine was measured in positive ion mode, Propofol and valproic acid in negative ion mode. Ions formed in the SESI source were detected by a high-resolution ($M/\Delta M \approx 22{,}000$) quadrupole time-of-flight mass spectrometer (AB SCIEX TripleTOF 5600+ system). Accumulation time was 10 s. A schematic of the experiment is shown in Figures 1a and S1. While the bulk of the sample consists of exhaled breath from the mouse in the sampling tube, there might also be contribution of transcutaneous gas exchange, which, based on values for cutaneous respiration should be below 1.5%[24]. For the sake of simplicity, we refer to the sample as “breath” throughout the text.

Plasma collection and determination of Ket plasma levels. In order to compare levels recorded in breath with plasma levels, animals were euthanized at the end of each breath measurement, and trunk blood was collected in heparinized Eppendorf tubes. Tubes were inverted multiple times and plasma was separated by centrifugation at 4°C for 10min at 5000xg. Plasma was frozen on dry ice and stored at -20°C until analysis. The levels of Ket and its metabolites were analyzed at the University of Bern using standard liquid chromatography-mass spectrometry methods.

Ketamine metabolite identification. Norketamine (NK) and Hydroxyketamine (HK) were identified both from their accurate mass and isotopic pattern (Figures S2 and S3) while hydroxynorketamine (HNK,

alcohol and phenol) and dehydronorketamine (DHNK) were identified only from the accurate mass, due to their lower intensity. It is noteworthy that, owing to the high mass accuracy and resolution of the HRMS instrument used, HNK (*m/z* 240.0787) is clearly separated and distinguished from the Ket isotope at *m/z* 240.0963 (Figure S3).

Propofol and valproic acid measurements. Propofol was identified based on its accurate mass, isotopic pattern, and on fragmentation experiments (Figure S10). Identification of valproic acid and its main metabolite (4-ene- valproic acid[25]) was based on its accurate mass and matching to simulated isotopic distribution.

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Keywords: Breath Analysis • Ketamine • Secondary Electrospray Ionization • High-Resolution Mass Spectrometry • Pharmacokinetics

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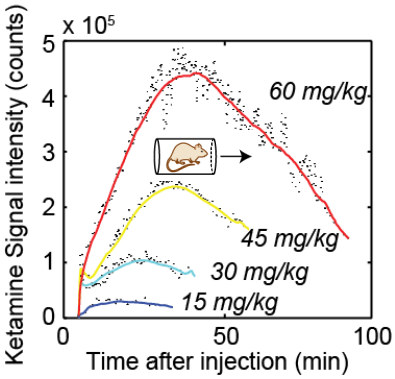
We present a novel, non-invasive *in-vivo* method based on secondary electrospray ionization mass spectrometry to determine pharmacokinetic (PK) properties of drugs compounds and their metabolites from the breath of a single mouse in real time. As an example, the PK of an antidepressant and anaesthetic drug, ketamine, and its metabolites was followed, with a time resolution of 10 s per data point.



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