Citation: CPT Pharmacometrics Syst. Pharmacol. (2019) 8, 107–117; doi:10.1002/psp4.12370

ARTICLE

Blood-Based Biomarkers of Quinpirole Pharmacology: Cluster-Based PK/PD and Metabolomics to Unravel the Underlying Dynamics in Rat Plasma and Brain

Willem J. van den Brink[1], Robin Hartman[1], Dirk-Jan van den Berg[1], Gunnar Flik[2], Belén Gonzalez-Amoros[1], Nanda Koopman[1], Jeroen Elassais-Schaap[1], Piet Hein van der Graaf[1,3], Thomas Hankemeier[1] and Elizabeth C.M. de Lange[1,*]

A key challenge in the development of central nervous system drugs is the availability of drug target specific blood-based biomarkers. As a new approach, we applied cluster-based pharmacokinetic/pharmacodynamic (PK/PD) analysis in brain extracellular fluid ($brain_{ECF}$) and plasma simultaneously after 0, 0.17, and 0.86 mg/kg of the dopamine $D_{2/3}$ agonist quinpirole (QP) in rats. We measured 76 biogenic amines in plasma and $brain_{ECF}$ after single and 8-day administration, to be analyzed by cluster-based PK/PD analysis. Multiple concentration-effect relations were observed with potencies ranging from 0.001–383 nM. Many biomarker responses seem to distribute over the blood-brain barrier (BBB). Effects were observed for dopamine and glutamate signaling in $brain_{ECF}$, and branched-chain amino acid metabolism and immune signaling in plasma. Altogether, we showed for the first time how cluster-based PK/PD could describe a systems-response across plasma and brain, thereby identifying potential blood-based biomarkers. This concept is envisioned to provide an important connection between drug discovery and early drug development.

Study Highlights

WHAT IS THE CURRENT KNOWLEDGE ON THE TOPIC?
☑ Metabolomic analysis provides an unbiased method of pharmacological biomarker discovery. Recently, cluster-based PK/PD modeling has been developed integrating PK/PD modeling and metabolomics analysis. There are no reliable blood-based biomarkers that reflect a specific drug effect in the brain.

WHAT QUESTION DID THIS STUDY ADDRESS?
☑ How cluster-based PK/PD modeling could be used to study biomarker responses across the BBB in order to identify blood-based biomarkers.

WHAT DOES THIS STUDY ADD TO OUR KNOWLEDGE?
☑ Multiple biogenic amines respond to the D_2 agonist QP in plasma and $brain_{ECF}$ showing different pharmacological patterns. Many of these potential biomarkers are transported over the BBB and five potential blood-based biomarkers were identified. Moreover, peripheral effects were found to propagate to the brain, putatively via the BBB.

HOW MIGHT THIS CHANGE DRUG DISCOVERY, DEVELOPMENT, AND/OR THERAPEUTICS?
☑ The discovery of blood-based biomarkers is envisioned to improve early CNS drug development by providing a method to monitor pharmacological effects in the brain.

One of the key challenges in central nervous system (CNS) drug development is the discovery of blood-based biomarkers that reflect the central response.[1,2] Such biomarkers enhance the evaluation of the proof of pharmacology of CNS drugs, which is crucial for successful drug development.[3] It is particularly important to dynamically evaluate the biomarker responses in relation to the systems pharmacokinetics (PKs) of the drug, given that the interaction between PKs and pharmacodynamics (PDs) typically is nonlinear and time-dependent.[4,5]

Although currently biomarker discovery is typically driven by the known pharmacological mechanisms, metabolomic fingerprinting is not limited to these pathways. Metabolomic analysis has revealed multiple new biochemical pathways in relation to drug responses.[6–11] Biomarker discovery for early CNS drug development is facing two challenges: (i) how could we evaluate the PK/PD interaction of an "omics" response; and (ii) how could we identify blood-based biomarkers that reflect drug effects in the brain?

[1]Division of Systems Biomedicine and Pharmacology, Leiden Academic Center for Drug Research, Leiden University, Leiden, The Netherlands; [2]Brains On-Line BV, Groningen, The Netherlands; [3]Certara QSP, Canterbury Innovation House, Canterbury, UK. *Correspondence: Elizabeth de Lange (ecmdelange@lacdr.leidenuniv.nl)
Received: September 26, 2018; accepted: October 15, 2018; published online on January 24, 2019. doi:10.1002/psp4.12370

One of the techniques being used in CNS biomarker discovery is intracerebral microdialysis. It is a well-established technique that has been successfully applied to study drug concentrations as well as drug response biomarkers in brain extracellular fluid ($brain_{ECF}$) to evaluate CNS PK and PD.[12–14] Therefore, microdialysis is the method of choice to dynamically evaluate a metabolomics fingerprint in $brain_{ECF}$ simultaneously upon CNS drug treatment. Such dynamical evaluation would improve the quantitative insights into systemwide responses (i.e., changes in biomarker concentrations), thereby shifting CNS drug development from an empirical toward a mechanistic discipline.[15,16]

In an earlier study, we have already shown that a cluster-based PK/PD evaluation of a metabolomic response in plasma reveals multiple dynamics underlying a system response upon treatment with remoxipride.[17] Although other methods exist to evaluate time-course metabolomics data, the cluster-based PK/PD methodology improves pharmacological interpretation (see ref. 17 for discussion). In the current study, we set out to extend this methodology with a simultaneous evaluation of a metabolomic response in both plasma and $brain_{ECF}$, using the selective dopamine $D_{2/3}$ receptor agonist quinpirole (QP) as paradigm compound with well-known PK/PD characteristics[18,19] to develop the methodology. Overall, the purpose is to develop a proof-of-concept methodology to provide insight into the biochemical responses of CNS drugs in $brain_{ECF}$ and plasma, combined with PK/PD modeling as a new approach to discovering blood-based biomarkers of central responses.

METHODS

Animals, surgery, and experiment

Animals. Animal studies were performed in agreement with the Dutch Law of Animal Experimentation and approved by the Animal Ethics Committee in Leiden, The Netherlands (study protocol DEC12247). For details on animals, surgery, and experiment, we refer to ref. 19.

Surgery. In short, male Wistar rats (n = 44) underwent surgery while anesthetized, to receive cannulas in the femoral artery and vein for blood sampling and drug administration, respectively. The microdialysis probe guides (CMA/12 Elite PAES, Schoonebeek, The Netherlands) and their dummy probes were implanted in both hemispheres of the caudate putamen that highly expresses D_2 receptors and has a large volume for implantation of a microdialysis probe. The probes (CMA/12 Elite PAES 4 mm, Schoonebeek, The Netherlands) were placed 24 hours before the experiment.

Experiment. The animals were subjected to an experiment on 2 days with 7 days in between (**Figure S1**). On the days of experiment, the rats were randomly assigned to receive 0 mg/kg (n = 12), 0.17 mg/kg (n = 16), or 0.86 mg/kg (n = 16) QP. Microdialysate samples were collected in anti-oxidant (10 μL 0.02 M formic acid/0.04% ascorbic acid in water) containing vials from –200 to 180 min (20-minute intervals, 1.5 μL/min, 120 minutes equilibration time). Blood samples were taken at –5, 5, 7.5, 10, 15, 25, 45, 90, 120, and 180 minutes and centrifuged to separate the plasma (1000 *g*, 10 minutes, 4°C). Samples were stored at –80°C until analysis. Between the experiment days, the same doses were administered subcutaneously.

Chemical analysis of the samples

As to develop a proof-of-concept methodology, two biogenic amine platforms were selected that had been validated for metabolomics analysis in both plasma and microdialysate samples. All compound identities were confirmed by high-resolution mass spectroscopy (MS) and identical retention times as authentic standards according to the proposed minimum standards of metabolomic analysis.[20]

Monoamine + metabolite analysis (platform A). A selection of plasma and microdialysate samples collected on experiment day 1 were analyzed by BrainsOnline (Groningen, The Netherlands; see refs. 21 and 22 for details). The samples were delivered on dry ice and stored at –80°C until analysis. After randomization of the samples, monoamines, and their metabolites (serotonin, 5-hydroxy indoleacetic acid, dopamine (DA), 3,4-hydroxyphenylacetic acid (DOPAC), homovanillic acid (HVA), glutamate, and glycine) were analyzed using the SymDAQ derivitization agent.[21,22] Data were calibrated and quantified using the Analyst data system (Applied Biosystems, Bleiswijk, The Netherlands) to report concentrations of the analytes (nM for all metabolites, except glutamate and glycine, which were reported in μM).

Biogenic amine analysis (platform B). The biogenic amines were analyzed in microdialysate and plasma samples of experiment on days 1 and 8 according to a previously described method.[23] Samples were randomized and amino acids and amines were derivatized by an Accq-tag derivatization strategy. Plasma samples (5 μL) were reduced with tris(2-carboxyethyl)phosphine and deproteinated by MeOH. Microdialysate samples (30 μL) were only reduced with tris(2-carboxyethyl)phosphine. The samples were dried under vacuum while centrifuged (9400 *g*, 10 minutes, room temperature), and reconstituted in borate buffer (pH 8.8) with 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate derivatization reagent. The reaction mixtures were injected (1 μL) into an ultraperformance liquid chromatography-tandem MS system, consisting of an Agilent 1290 Infinity II LC system, an Accq-Tag Ultra column, and a Sciex Qtrap 6500 MS. The peaks were assigned using Sciex MultiQuant software version 3.0.2, integrated, normalized for their internal standards, and corrected for background signal. Only compounds with a QC relative SD under 30% were reported to assure the quality of the data.

Data analysis

Pharmacokinetic model. The PK model has been published previously and described the free QP concentrations in plasma and $brain_{ECF}$ with QP doses ranging from 0.17–2.14 mg/kg.[19] The visual predictive check and external validation have been added as **Figures S2 and S3**.

Pharmacodynamic models. A PD model was developed for every single metabolite (hereafter called biomarkers) using a population approach in NONMEM version 7.3.0 using subroutine ADVAN13. The interindividual variability around the parameters and the residual error were described by an exponential distribution (**Supplementary Eqs. S1 and S2**). A combination of submodels was

evaluated for each single biomarker consisting of (i) a straight baseline, an exponential decay, or a linear slope model (**Supplementary Eq. S3**); (ii) a linear or a sigmoid maximum effect (E_{max}) concentration-response model; (iii) a transit or no transit compartment model; and (iv) a turnover or a pool model (**Supplementary Eqs. S4–S7**). In addition, a model with no drug response function was evaluated (**Supplementary Eq. S8**). The models were selected on basis of the objective function value (OFV; χ^2 test, $P < 0.05$), the condition number, successful convergence, and visual evaluation of goodness-of-fit plots.

Exploration of target site. For biomarkers showing a response in either plasma or $brain_{ECF}$, the site with the response was identified as effect target site. In case a biomarker showed a response both in plasma and $brain_{ECF}$, two PD models were developed. One model (A) with QP in $brain_{ECF}$ driving the biomarker response in $brain_{ECF}$. The biomarker response in plasma was linked to the $brain_{ECF}$ biomarker response by a linear or a nonlinear brain transport model following Michaelis Menten kinetics (**Supplementary Eq. S9**). In another model (B), QP in plasma was driving the biomarker response in plasma. The biomarker response in $brain_{ECF}$ was then linked to the plasma biomarker response following the brain transport model (**Supplementary Eq. S9**). The model with the lowest Akaike Information Criterion (AIC) was selected as the best model. This was done by subtracting the AIC of the "$brain_{ECF}$ target site model" from that of the "plasma target site model" to calculate the ΔAIC. A negative ΔAIC indicated plasma as the target site of effect, whereas a positive ΔAIC suggested $brain_{ECF}$ as the target site of effect.

Clustering. The longitudinal biomarker responses were simulated for their determined target site and subsequently clusters of the dynamical pharmacological responses were identified in plasma and $brain_{ECF}$ using *k*-means clustering (R version 3.3.1, package "stats," function "kmeans"). The number of clusters was selected in two steps. First, an elbow plot depicting the number of clusters against the within-cluster sum of squares was used to identify the range of the potential number of clusters to be used in the cluster-based PK/PD model. Second, a cluster-based PK/PD model was developed describing the PK/PD profile of the clusters for each scenario. The AIC was used to select the model with the optimal number of clusters. Subsequently, a stepwise parameter sharing procedure was applied as previously described.[17] In short, a single parameter (e.g., half-maximal effective concentration (EC_{50})) was estimated for multiple clusters and evaluated by the change in OFV (χ^2 test, $P < 0.05$) to determine whether this was statistically different from a model with separate parameters. If no difference was found, the shared parameter was kept in the model.

Significance score calculation. The cluster-based model was compared to a model with no drug effect model included (i.e., assuming no effect of QP). A significance score was calculated by the change in OFV corrected for the degrees of freedom with a Bonferroni-corrected significance threshold of $\alpha = 0.01$ (**Supplementary Eq. S10**). A significance score > 0 reflects a significant effect of QP on a biomarker response.

Effect of 8-day QP administration

Basal biomarker levels ($t = 0$) in both $brain_{ECF}$ and plasma at experiment day 1 and experiment day 8 were compared using two-way analysis of variance with interaction between dose and experiment day. The Tukey-honest significant difference test was used for *post hoc* analysis. $Brain_{ECF}$ basal biomarker levels were averaged per animal, given that there were 4–6 baseline samples for each animal. For the biomarkers that revealed a significant change with experiment day, a covariate analysis was performed in the single biomarker models by estimating a separate baseline parameter per combination of the treatment group and the day of the experiment. Only if the covariate analysis revealed a difference, the effect was considered significant.

RESULTS

Exploration of the target site of effect

A total of 7 metabolites were reported from platform A, whereas 54 metabolites were found having an QC relative SD below 30%. From those metabolites, the combined PK/PD analysis in plasma and $brain_{ECF}$ revealed 23 biomarkers primarily responding to QP in plasma, and 15 biomarkers primarily affected by QP in the brain (**Table 1, Figure 1**). DL-3-aminobutyric acid and serotonin could only be measured in plasma, whereas L-glutamine could only be measured in $brain_{ECF}$. From all the biomarkers that reflected an effect of plasma QP, 19 showed a net transport to the $brain_{ECF}$. Inversely, five biomarkers exhibited a net transport from $brain_{ECF}$ into plasma, being indicated as potential blood-based biomarkers of drug effect in the brain. The intercompartmental transport rates between plasma and $brain_{ECF}$ of many biomarkers were described by nonlinear Michaelis-Menten kinetics (**Table 1**).

Clustered response patterns in $brain_{ECF}$ and plasma

A total of seven clusters of dynamical biomarker responses in $brain_{ECF}$ were selected (**Figure S4, Table 2**). Using parameter sharing, it was observed that the biomarkers responded with either a high or a low potency (EC_{50} = 0.01 nM or EC_{50} = 122 nM; **Table 3, Data S1, Figure 2**). The turnover of these biomarkers was low (0.031–0.056 min^{-1}) or high (0.13–0.44 min^{-1}) as a reflection of their different time-courses (**Figure 3, Table 3**). The responses in plasma were also separated into seven clusters (**Figure S4, Table 2**) described by models with transit compartment models (clusters 1 and 4), pool models (clusters 5 and 6), and turnover models (clusters 2, 3, and 7; **Table 3, Data S2**). Thus, the time courses of the biomarker responses in plasma were different among the clusters, not only indicated by the turnover rates being low (0.057–0.060 min^{-1}) or high (0.11 min^{-1}), but also by the fact that their description needed different dynamic models (**Figure 3, Table 3**). A wider variety of potency parameter estimates was identified in plasma as compared to $brain_{ECF}$: 0.01 nM, 17.2 nM, and 113–383 nM (**Table 3, Figure 2**). Moreover, the direction of response was both up (clusters 1 and 4) and down (clusters 2, 3, and 5–7). The responses in $brain_{ECF}$ and plasma were well described by the cluster-PK/PD models (**Figure 3, Figure S5**).

Table 1 Overview of biogenic amines and their target site that showed a response upon QP treatment

Biomarker	Target site	ΔAIC	Brain transport
Platform A		(BrainsOnline)	
DA	$Brain_{ECF}$	–	No
DOPAC	$Brain_{ECF}$	–	No
HVA	$Brain_{ECF}$	–	No
Glycine	Plasma	−56.216	Yes – $NonLin_{P\rightarrow B}$
5-HIAA	Plasma	–	No
L-Glutamic acid	Plasma	–	No
Platform B		(BMFL)	
L-Phenylalanine	Plasma	−75.811	Yes – $NonLin_{B\rightarrow P}$
L-Valine	Plasma	−73.682	Yes – $NonLin_{B\rightarrow P}$
L-Methionine sulfoxide	Plasma	−55.917	Yes – $NonLin_{P\rightarrow B}$
Taurine	Plasma	−48.638	Yes – $NonLin_{B\rightarrow P}$
S-Methylcysteine	Plasma	−46.564	Yes – Linear
L-Alpha-aminobutyric acid	Plasma	−40.634	Yes – $NonLin_{P\rightarrow B}$
L-Asparagine	Plasma	−37.597	Yes – $NonLin_{B\rightarrow P}$
L-Alanine	Plasma	−35.086	Yes – $NonLin_{P\rightarrow B}$
Gamma-L-glutamyl-L-alanine	Plasma	−33.872	Yes – $NonLin_{P\rightarrow B}$
L-Threonine	Plasma	−31.734	Yes – Linear
L-Methionine	Plasma	−24.946	Yes – Linear
L-Histidine	Plasma	−24.715	Yes – Linear
L-Arginine	Plasma	−24.469	Yes – $NonLin_{P\rightarrow B}$
L-Isoleucine	Plasma	−13.582	Yes – $NonLin_{B\rightarrow P}$
Glycine	Plasma	−12.572	Yes – Linear
Homocysteine	Plasma	−10.954	Yes – Linear
L-Serine	Plasma	−8.129	Yes – Linear
Citrulline	Plasma	−5.407	Yes – $NonLin_{B\rightarrow P}$
L-Leucine	Plasma	−2.462	Yes – $NonLin_{B\rightarrow P}$
DL-3-aminoisobutyric acid	Plasma	–	N.A.
Histamine	Plasma	–	No
L-Glutamic acid	Plasma	–	No
L-Homoserine	Plasma	–	No
Methionine sulfone	Plasma	–	No
Serotonin	Plasma	–	N.A.
L-Proline	$Brain_{ECF}$	41.574	Yes – $NonLin_{B\rightarrow P}$
N6,N6,N6-Trimethyl-L-lysine	$Brain_{ECF}$	27.282	Yes – $NonLin_{B\rightarrow P}$
Hydroxylysine	$Brain_{ECF}$	8.103	Yes – Linear
L-Lysine	$Brain_{ECF}$	4.747	Yes – $NonLin_{B\rightarrow P}$
L-4-hydroxy-proline	$Brain_{ECF}$	1.111	Yes – $NonLin_{B\rightarrow P}$
Homocitrulline	$Brain_{ECF}$	0.261	Yes – $NonLin_{B\rightarrow P}$
3-Methoxytyramine	$Brain_{ECF}$	–	No
5-Hydroxy-L-tryptophan	$Brain_{ECF}$	–	No
Cystathionine	$Brain_{ECF}$	–	No
Gamma-aminobutyric acid	$Brain_{ECF}$	–	No
L-2-aminoadipic acid	$Brain_{ECF}$	–	No
L-Glutamine	$Brain_{ECF}$	–	N.A.
L-Tryptophan	$Brain_{ECF}$	–	No
L-Tyrosine	$Brain_{ECF}$	–	No
Ornithine	$Brain_{ECF}$	–	No
Putrescine	$Brain_{ECF}$	–	No
Sarcosine	$Brain_{ECF}$	–	No

The Delta Akaike Information Criterium (ΔAIC) indicates the target site (see Methods). In addition, the type of brain transport is indicated (yes, no or not available (N.A.)). $P \rightarrow B$ and $B \rightarrow P$ stand for plasma-to-brain and brain-to-plasma, respectively. Only biomarkers presented in black showed a significant response in the cluster models

$Brain_{ECF}$, brain extracellular fluid; DA, dopamine; DOPAC, 3,4-hydroxyphenylacetic acid; HIAA, 5-hydroxy indoleacetic acid; HVA, homovanillic acid; QP, quinpirole.

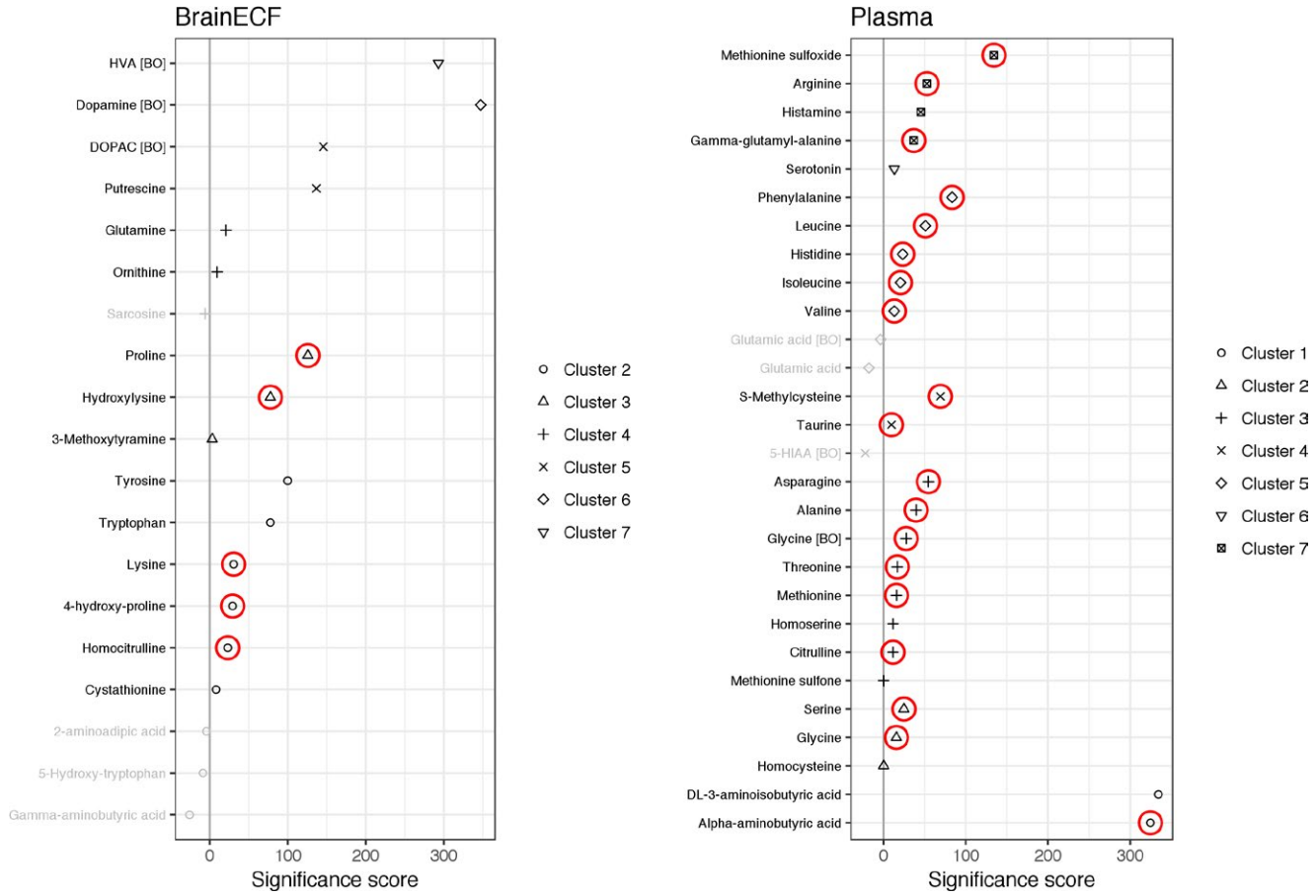


Figure 1 Significance score of metabolites responding to quinpirole (QP) in brain extracellular fluid ($brain_{ECF}$) (left) and plasma (right) indicating their potential as a biomarker of the QP systems effect. The gray line marks the significance threshold; metabolites to the right of the line were significantly affected by QP. The red circles indicate the metabolites that distribute from $brain_{ECF}$ to plasma and vice versa. *Cluster 1 of $brain_{ECF}$ was excluded from this figure because no effect was observed. BO refers to the amines analyzed by BrainsOnline. DOPAC, 3,4-hydroxyphenylacetic acid; HVA, homovanillic acid.

Effect of QP on the dopamine pathway

DA, DOPAC, and HVA, the key constituents of the DA pathway, were decreased in $brain_{ECF}$ upon QP treatment. Whereas the *in vivo* potency was found to be similar for these biomarkers (122 nM), the maximal inhibition values (DA: 67%, DOPAC: 41%, and HVA: 60%) and the turnover rates (DA: 0.44 min^{-1}, DOPAC: 0.13 min^{-1}, and HVA: 0.031 min^{-1}) were different (**Table 3, Figure 2**). No responses of QP treatment were observed for DA and HVA in plasma, whereas DOPAC could not be measured in plasma due to assay lower limit of detection of 50 nM.

Effect of QP on other pathways in $brain_{ECF}$

In $brain_{ECF}$, QP was found to interact with the polyamine metabolism[24] (ornithine, putrescine), the proline metabolism (proline, L-4-hydroxyproline), neurotransmitter precursors (tryptophan and tyrosine), and lysine metabolism (lysine, hydroxylysine; **Table 1, Figure 1**).

Effect of QP on metabolic pathways in plasma

The systemic response on amino acid metabolism in plasma indicated interactions between QP and the branched chain amino acid (BCAA) metabolism (leucine, isoleucine, and valine), neurotransmitter synthesis (phenylalanine), serine-glycine-threonine metabolism (serine, glycine, threonine), and histamine metabolism (histidine, histamine; **Table 1, Figure 1**). Furthermore, alpha-aminobutyric acid and DL-3-aminoisobutyric acid strongly responded to QP treatment (**Table 1, Figure 1**).

Effect of 8-day QP administration on basal biomarker levels

Eight-day QP administration did not result in significant changes in basal $brain_{ECF}$ biomarker levels but showed a significant change in plasma levels of alpha-aminobutyric acid and DL-3-aminoisobutyric acid after 0.17 mg/kg ($P < 0.05$), but not after 0.86 mg/kg QP ($P > 0.05$; **Figure 4**). However, including the interaction between treatment and day as a covariate in the PK/PD models for these biomarkers did not result in a significant improvement of the model ($P > 0.05$), potentially related to the lack of a dose-response relation (**Figure 4**).

DISCUSSION

In this study, we aimed for combining metabolomics in $brain_{ECF}$ and plasma as an extension to the earlier developed

Table 2 Determination of optimal number of clusters in plasma and $brain_{ECF}$ using the AIC

Plasma		BrainECF	
# Clusters	**AIC**	**# Clusters**	**AIC**
4	65500.76	6	78140.64
5	64991.03	**7**	**76518.12**
6	64966.79	8	76523.49
7	**64876.42**	9	78319.55
8	66314.62	10	76535.81

In bold are the selected number of clusters.
AIC, Akaike information criterion; $brain_{ECF}$, brain extracellular fluid.

Table 3 Parameter estimates of the cluster models

Plasma		$Brain_{ECF}$	
Parameter	**Estimate (RSE)**	**Parameter**	**Estimate (RSE)**
Cluster 1[a]			
E_{max} (%)	4650 (41.1%)		
EC_{50} (nM)	383 (54.3%)		
k_{out} (min^{-1})	0.035 (42.3%)		
$k_{transit}$ (min^{-1})	0.044 (33.1%)		
$n_{transit}$	8.3 (19.2%)		
Cluster 2			
I_{max} (%)	−20 (30.1%)	I_{max} (%)	−20 (6.1%)
IC_{50} (nM)	113 (98.5%)	IC_{50} (nM)	0.001 (fix)
k_{out} (min^{-1})	0.057 (38.3%)	k_{out} (min^{-1})	0.056 (27.9%)
Cluster 3			
I_{max} (%)	−20 (30.1%)	I_{max} (%)	−29 (7.1%)
IC_{50} (nM)	17.2 (50.6%)	IC_{50} (nM)	0.001 (fix)
k_{out} (min^{-1})	0.11 (12.2%)	k_{out} (min^{-1})	0.13 (13.3%)
Cluster 4			
E_{max} (%)	363 (67.5%)	I_{max} (%)	−15 (13.5%)
EC_{50} (nM)	113 (98.5%)	IC_{50} (nM)	0.001 (fix)
k_{out} (min^{-1})	9.58 (104%)	k_{out} (min^{-1})	0.14 (32.7%)
$k_{transit}$ (min^{-1})	0.0052 (46.8%)		
$n_{transit}$	1.79 (17.9%)		
Cluster 5			
I_{max} (%)	−41 (14.6%)	I_{max} (%)	−41 (9.0%)
IC_{50} (nM)	339 (32.8%)	IC_{50} (nM)	122 (51.4%)
k_{out} (min^{-1})	0.11 (12.5%)	k_{out} (min^{-1})	0.13 (13.3%)
k_{rel} (min^{-1})	0.018 (27.5%)		
Cluster 6			
I_{max} (%)	−90 (0.3%)	I_{max} (%)	−67 (4.9%)
IC_{50} (nM)	0.001 (fix)	IC_{50} (nM)	122 (51.4%)
k_{out} (min^{-1})	0.10 (18.4%)	k_{out} (min^{-1})	0.44 (47.9%)
k_{rel} (min^{-1})	0.89 (19.7%)		
Cluster 7			
I_{max} (%)	−41 (6.4%)	I_{max} (%)	−60 (9.3%)
IC_{50} (nM)	17.2 (50.6%)	IC_{50} (nM)	122 (51.4%)
k_{out} (min^{-1})	0.060 (13.5%)	k_{out} (min^{-1})	0.031 (28.9%)

$brain_{ECF}$, brain extracellular fluid; EC_{50}, half-maximal effective concentration; E_{max}, maximum effect; IC_{50}, half-maximal inhibitory concentration; I_{max}, maximum unbound systemic concentration.
[a]Cluster 1 of $brain_{ECF}$ was excluded from this table because no dose-response was observed. Consequently, parameter estimates were not informative.

cluster-based PK/PD modeling approach (see ref. 17), in order to obtain insight into the systems-response, as well as to explore the target site of the effect upon CNS drug administration. By evaluating time-resolved metabolomics in both $brain_{ECF}$ and plasma, we revealed a few potential blood-based biomarkers reflecting effects in $brain_{ECF}$. Interestingly, it was also observed that many biochemical responses of QP have their main origin in the periphery rather than in the $brain_{ECF}$. Additionally, the integration of time-resolved metabolomics analysis with cluster-based PK/PD revealed the diverse dynamical responses of biogenic amines and amino acids in $brain_{ECF}$ and plasma upon administration of the $D_{2/3}$ agonist QP. Indeed, the quantitative characterization of the systemwide biomarker responses showed a variety of *in vivo* potency and maximal response values in both $brain_{ECF}$ and plasma. Furthermore, in addition to the dopamine pathway, several other biochemical pathways were potentially affected by QP. Finally, our study showed no response of 8-day administration on biogenic amine and amino acid levels. Here, we will discuss each of these observations to finish the discussion with the limitations of our study and suggestions for further investigations.

Exploration of the target site and identification of blood-based biomarkers

It is a great challenge to identify blood-based biomarkers that reflect neurochemical responses in the brain. Often, these measurements are done at a single timepoint limiting the identification of causality. In the current study, we were able to use the time-delay between the $brain_{ECF}$ and plasma biomarker responses to identify the potential causal relationship between them. With this, we assume that the delay represents transport of a biomarker over the BBB. The BBB has multiple transport systems that transport biogenic amines and amino acids, for example, the large neutral amino acid transporter 1 (for transport of e.g., glutamine, tyrosine, and tryptophan), the cationic amino acid transporter 1 (for transport of arginine and lysine), or the serotonin transporter (for transport of serotonin).[25,26] These transport systems exist at both the luminal and abluminal site of the BBB, whereby biogenic amines and amino acids can be transported from plasma to brain and vice versa. It is, therefore, likely that the parallel responses in plasma are, at least partially, explained by BBB transport.

Interestingly, the number of biogenic amines transported from $brain_{ECF}$ to plasma was lower than those transported from plasma to brain (**Figure 1, Table 1**). This observation suggests, first of all, that even if a drug does not cause a direct response in the brain (e.g., because there is no drug exposure in the brain), biochemical responses may propagate from plasma to $brain_{ECF}$ and cause secondary responses. Second, the observed asymmetry confirms the well-known difficulty of finding blood-based markers reflective of drug responses in $brain_{ECF}$.

Nevertheless, five potential blood-based biomarkers reflected a response in $brain_{ECF}$ (**Table 1, Figure 1**). Importantly, four of them showed nonlinear transport over the BBB. This is relevant when evaluating blood-based biomarkers as a surrogate for an effect in $brain_{ECF}$; a nonlinear

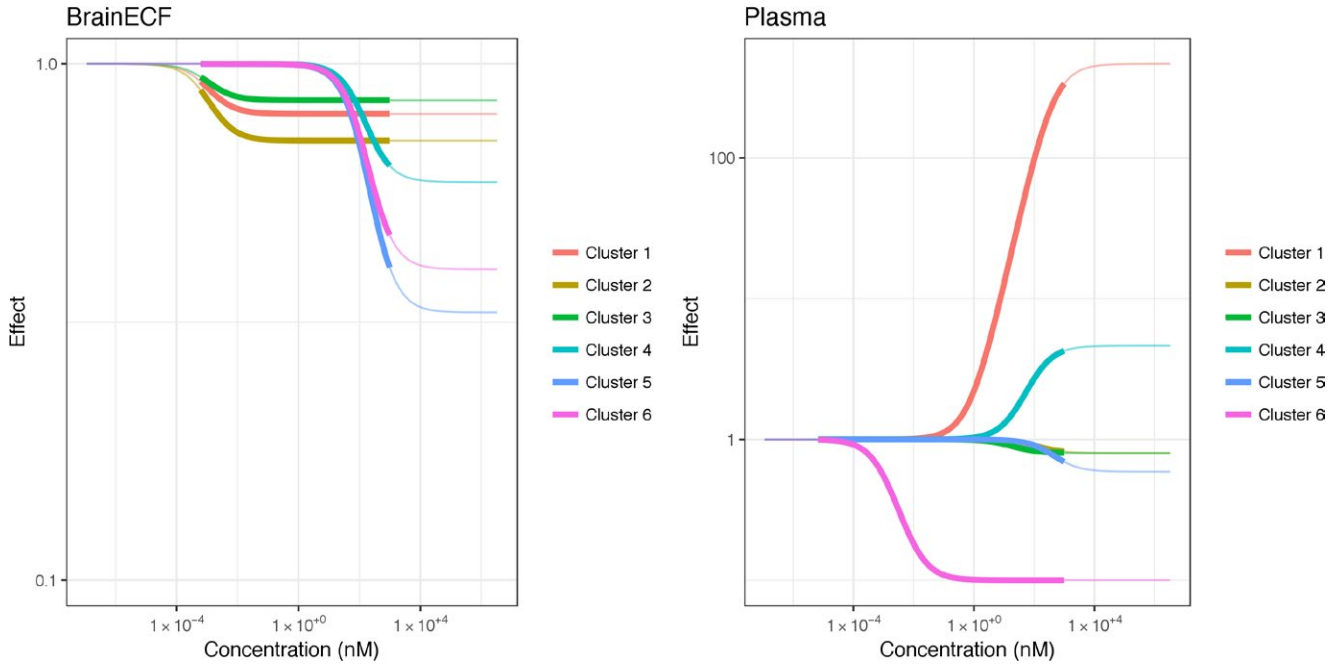


Figure 2 An overview of the concentration-effect relations that underlie the systems responses in brain extracellular fluid ($brain_{ECF}$) (left) and plasma (right). Thick line parts represent the range of observed biomarker concentrations. Cluster 1 was excluded for $brain_{ECF}$ because no effect was observed.

relation between drug concentration and plasma biomarker response may reflect nonlinear BBB transport and, hence, affect the estimation of the E_{max} parameter. Therefore, in order to understand the dynamics of the blood-based biomarker response in a clinical context, it is recommended to first determine the relationship between the plasma and $brain_{ECF}$ biomarker response in a preclinical setting with possibilities of simultaneous sampling of plasma and $brain_{ECF}$ in a continuous manner.

A diverse pharmacological range of PK/PD clusters

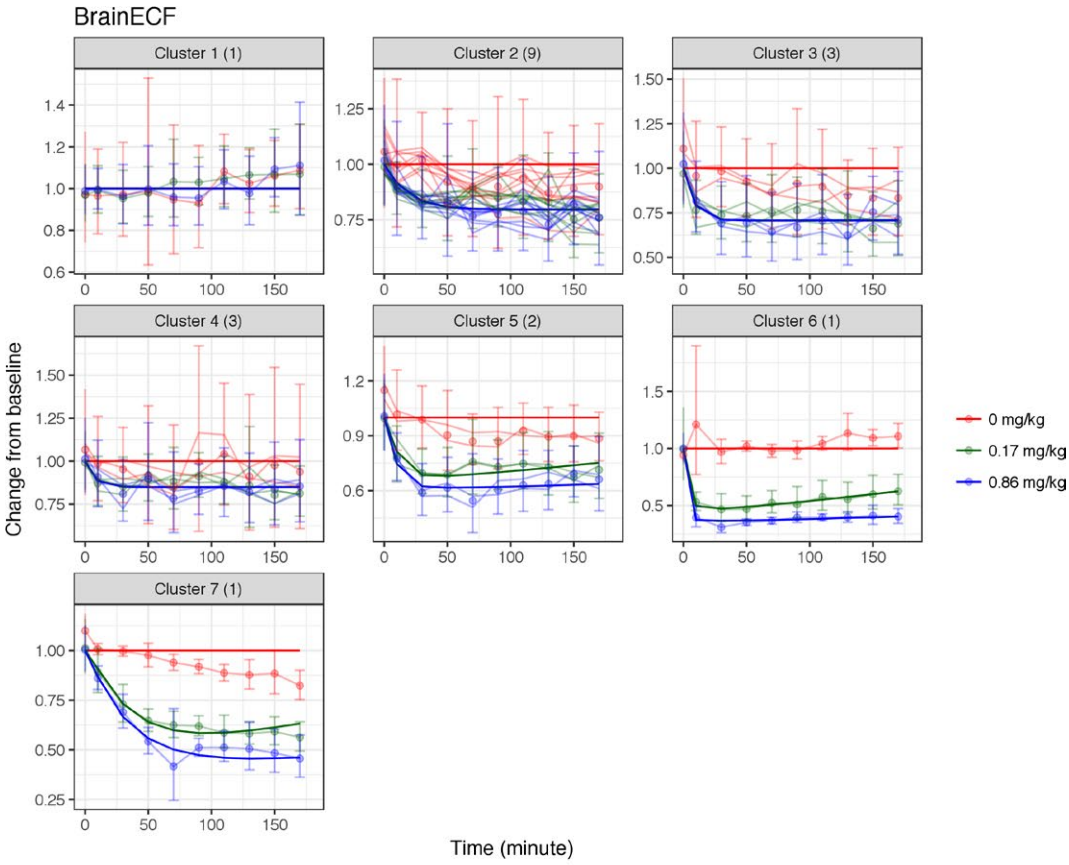
Both the $brain_{ECF}$ and plasma biomarker responses were combined into seven clusters. These clusters represented different pharmacological characteristics (e.g., the potencies in $brain_{ECF}$ ranged from 0.01–122 nM), whereas those in plasma ranged from 0.01–383 nM (**Table 3**). An important question is what these pharmacological parameters represent. First of all, the cluster-based PK/PD approach improved the robustness of the model by a dramatic reduction in the number of parameters without compromising the quality of the model. Second, although it is not possible to determine whether the potency differences are related to off-target effects or different signal transduction efficiencies (see ref. 19 for discussion), the cluster-based PK/PD model can define a therapeutic range on basis of a system response in plasma and $brain_{ECF}$. Elements of this model may be selected as input for mechanistic systems pharmacology models. For example, the dopamine pathway is represented by DA, DOPAC, and HVA, which all have an estimated potency of 122 nM, whereas the turnover rates differ (**Table 3**). Thus, it seems that they are driven by the same drug-target interaction, with no differences in signal transduction efficiency. This confirms what we know from a biochemical point of view, and, indeed, these biomarkers have been described by a mechanistic systems pharmacology model in an integrated manner.[13]

The effects of QP on multiple pathways

The QP seemed to have an overall inhibiting response on multiple biogenic amine pathways. First of all, the DA metabolism in the $brain_{ECF}$ was inhibited, which could be explained by the response of QP on the D_2 autoreceptors located on the presynaptic neuron.[27] Moreover, QP reduced peripheral phenylalanine concentrations, thereby possibly lowering the brain levels of phenylalanine and tyrosine that constitute the basis of the DA metabolism. Second, although QP did not significantly affect cerebral glutamate levels, glutamate signaling may be inhibited by QP, given that glycine, serine, proline, and putrescine levels in $brain_{ECF}$ were decreased, all presumably influencing the N-methyl-D-aspartate receptor in a direct or indirect manner.[28–30]

Furthermore, the reduction of the BCAA levels and the increase of DL-3-aminoisobutyric acid in plasma may both be associated with increased activity of the animals. BCAA levels were found negatively correlated with activity,[31] whereas DL-3-aminoisobutyric acid was observed positively associated with the level of activity.[32] Indeed, QP does induce locomotion as a measure of increased activity and movement,[33] and the modified levels of BCAA and DL-3-aminoisobutyric acid in our study may be a reflection of that.

Finally, the reduction of histidine and histamine in plasma may reflect an inhibitory effect of QP on the immune system. Histamine is directly released from dendritic cells,


BrainECF
Cluster 1 (1)
Cluster 2 (9)
Cluster 3 (3)
Cluster 4 (3)
Cluster 5 (2)
Cluster 6 (1)
Cluster 7 (1)
Change from baseline
Time (minute)
0 mg/kg
0.17 mg/kg
0.86 mg/kg

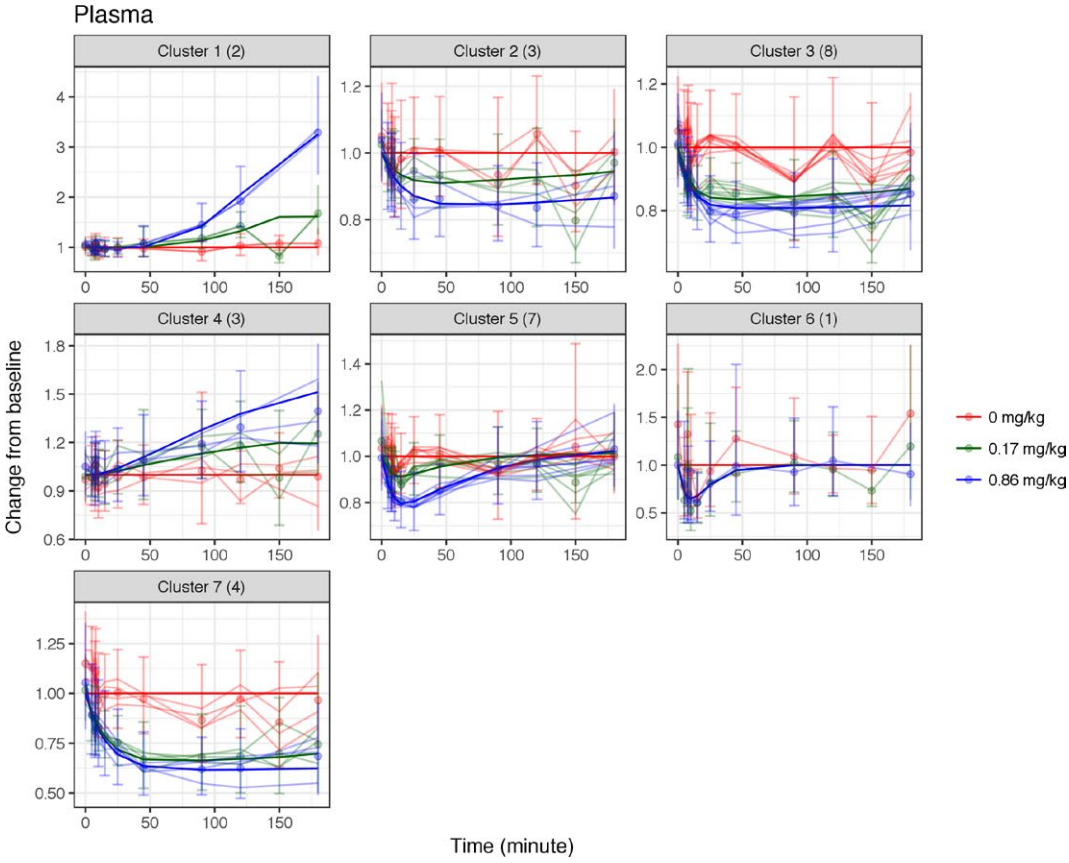

Plasma
Cluster 1 (2)
Cluster 2 (3)
Cluster 3 (8)
Cluster 4 (3)
Cluster 5 (7)
Cluster 6 (1)
Cluster 7 (4)
Change from baseline
Time (minute)
0 mg/kg
0.17 mg/kg
0.86 mg/kg

Figure 3 Goodness-of-fit of the cluster responses as change from baseline in brain extracellular fluid (top) and plasma (bottom). Dots and error bars mark the geometric mean ± SD of the observed cluster responses, light lines represent the geometric mean of the single metabolite responses, and dark lines show the predicted cluster responses. The facet labels show the number of metabolites between the parentheses.

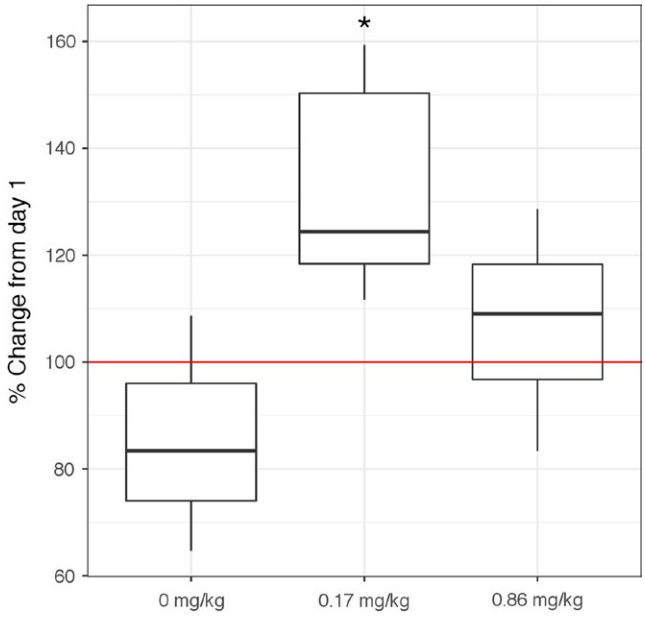


Figure 4 Relative change of L-alpha-aminobutyric acid levels in plasma after 8-day administration as compared to a single administration. *Denotes a significant effect with $P < 0.05$.

macrophages, and neutrophils upon production from histidine by the enzyme histidine decarboxylase.[34] Interestingly, DA receptors are expressed in various immune cells, such as dendritic cells, neutrophils, and natural killer cells,[35] indicating a potential mechanism through which QP may have influenced the histamine metabolism.

The effects of 8-day QP administration

Interestingly, although there was a significant response upon 8-day administration of QP in PK/PD parameters describing the neuroendocrine response,[19] no significant impact on basal biomarker levels was identified in the current study, although DA, DOPAC, and HVA were only analyzed for experiment day 1. Our hypothesis to see an effect after 8 days was based on a study in which behavioral tolerance and sensitization were observed within a period of 1 week after administration of a D_2 agonist in mice.[36] A possible explanation for the lack of an 8-day response in our study could be that the biological systems that underlie the amino acid and biogenic amine responses have greater flexibility than the neuroendocrine system in adapting to perturbations, such as QP administration. Longer studies should be performed to provide conclusive evidence of absence of the long-term effects of QP on biogenic amines.

Limitations of the current study and future investigations

We are aware of the limitations of this study. First of all, although the results in our study strongly indicate a systemwide response for the $D_{2/3}$ receptor agonist QP, it should be confirmed by using other D_2 agonists whether the observed responses are related to dopaminergic activity, and to which receptor subtype they are related. Such analysis would give insights into drug-class specific systemwide responses. For example, a multivariate analysis of several antipsychotic D_2 receptor antagonists showed large neurochemical and behavioral overlap of clozapine with 5-HT_{2a} antagonists, but not haloperidol.[37] Ultimately, the cluster-based PK/PD approach may link *in vitro* and *in vivo* characterizations of drug-class related pharmacology by connecting the pattern of *in vivo* potencies to *in vitro* affinities.

Second, although the analytical platforms that have been used in the current study are well-developed with proven robustness,[21,23] glycine measured by platform A was described by cluster 3 dynamics, whereas the glycine response as analyzed by platform B was closer to the cluster 2 pattern (**Figure 1**). Interlaboratory reproducibility is currently a topic of investigation in the field of metabolomics, although early research suggests good robustness of metabolomics platforms toward this type of variation.[38] An explanation could be nonlinearity of the apparatus response given the fact that platform B provided response ratios (analyte peak area/internal standard peak area), whereas platform A presented concentrations.

Third, although not only biogenic amines and amino acids are expected to respond to QP, we were limited by sample volume of the microdialysates. It would be valuable to extend the current approach with multiple platforms integrated to obtain a comprehensive insight into the systemwide effects of CNS drugs. Fortunately, the microdialysis-metabolomics technology is rapidly evolving, requiring lower sample volumes for metabolomics analysis.[39,40] Furthermore, to counteract the high attrition rates in CNS drug development, it will be important to accurately monitor the pharmacology in early clinical drug development.[3] Such monitoring needs accessible biomarkers that can be obtained from the blood, for example. The combined microdialysis-metabolomics technology is envisioned valuable and relatively low-cost to develop specific biomarker panels for CNS drugs (or drug classes).

Finally, all $brain_{ECF}$ measurements were made in the striatum. To gain insight into the higher hierarchy of the brain, the brain circuitry, it is essential to do measurements in multiple brain regions that are relevant to the drugs' mechanism of action. Indeed, CNS diseases and treatment responses are determined by the balance among signaling of multiple neurotransmitters in multiple regions.[41–43] Moreover, in some disease conditions, cerebral spinal fluid (CSF) may provide a good alternative as a sampling site if plasma sampling does not provide biomarkers of central effect. Moreover, to gain a good understanding of the kinetics of endogenous compounds, such as biogenic amines, it will be important to include CSF. Indeed, this

has been shown for physiology-based PK models describing drug concentrations in plasma, $brain_{ECF}$, and CSF.[44–46] The addition of multiple brain regions to a cluster-based PK/PD model is, therefore, envisioned to further elucidate the systems PDs of CNS drugs.

CONCLUSION

CNS drug development is challenged by low success rates and high development costs. Biomarker-driven drug development is seen as a logical step to improve these success rates, and metabolomics holds great promise in this regard. It provides a relatively low-cost method to comprehensively screen for drug response biomarkers. In this study, we showed for the first time how time-resolved metabolomics analysis in combination with cluster-based PK/PD describes the diverse dynamical patterns in $brain_{ECF}$ and plasma in terms of pharmacological parameters (e.g., E_{max} and EC_{50}) to evaluate multibiomarker (eventually systemswide) CNS drug effects. Moreover, our approach also enables to identify the potential target site of effect, as well as to identify blood-based biomarkers that are reflective of drug responses in $brain_{ECF}$. Although the identified biomarkers warrant validation, further application and development of this method are envisioned to provide an important connection between drug discovery and early drug development.

Supporting Information. Supplementary information accompanies this paper on the *CPT: Pharmacometrics & Systems Pharmacology* website (www.psp-journal.com).

Figure S1. Schematic presentation of the study design.

Figure S2. Visual predictive check of the pharmacokinetic model describing free quinpirole concentrations in plasma and $brain_{ECF}$ of rats after intravenous administration of 0.17, 0.43, 0.86, and 2.14 mg/kg quinpirole.

Figure S3. External validation of the pharmacokinetic model describing free quinpirole concentrations in plasma and $brain_{ECF}$ of rats after intravenous administration of 1.0 mg/kg quinpirole.

Figure S4. Elbow plots for the clustering of $brain_{ECF}$ (left) or plasma (right) responses.

Figure S5. Goodness-of-fit of the cluster models on the baseline corrected single metabolite levels in $brain_{ECF}$ (top) and plasma (bottom).

Supplementary Eq. S1. Interindividual and residual variability.

Data S1. Model_code cluster-PK/PD model brain.

Data S2. Model_code cluster-PK/PD model plasma.

Funding. No funding was received for this work.

Conflict of Interest. As Editor-in-Chief of *CPT: Pharmacometrics & Systems Pharmacology*, Piet H. van der Graaf was not involved in the review or decision process for this article.

Author Contributions. W.vdB., J.E.S., P.H.vdG., and E.C.M.L. wrote the manuscript. W.vdB., T.H., and E.C.M.L. designed research. W.B., G.H., D.J.vdB., G.F., B.A.G., N.K., and A.C.H. performed research. W.B. analyzed the data.

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