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Quantitative Detection of Nucleoside Analogs by Multi-Enzyme Biosensors using Time-Resolved Kinetic Measurements

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Graphical abstract

Keywords

biosensor; data fusion; deoxynucleoside kinase; nucleoside analog; therapeutic dosage monitoring

Fast, simple and cost-effective methods for detecting and quantifying pharmaceutical agents in patients are highly sought after to replace equipment and labor-intensive analytical procedures. The development of new diagnostic technology including portable detection devices also enables point-of-care by non-specialists in resource-limited environments. We have focused on the detection and dose monitoring of nucleoside analogs used in viral and cancer therapies. Using deoxyribonucleoside kinases (dNKs) as biosensors, our chemometric model compares observed time-resolved kinetics of unknown analytes to known substrate interactions across multiple enzymes. The resulting dataset can simultaneously identify and quantify multiple nucleosides and nucleoside analogs in complex sample mixtures.

Traditional analytical methods for detecting pharmaceutical agents are based on gas or liquid chromatography coupled to mass spectrometry.^[1,2] Unfortunately, these methods are somewhat limited for the detection of multiple analytes, requiring labor-intensive sample preparation and column conditions for each species. Current detection can also be

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prohibitive in applications such as clinical monitoring, often requiring large volumes of blood (5-25 mL) to detect multiple analytes, a specific concern among pediatric patients.

A potential solution is to leverage the inherent selectivity of enzymes to directly detect analytes in unmodified samples. Enzymes are routinely used as analytical reagents in biological assays, offering a quantitative method for monitoring substrate conversion. Relative to traditional chromatographic methods, enzyme-based biosensors are simple and specific systems with typically rapid assay times and lower equipment costs.^[3] An example for enzyme-based biosensors is the detection of neurotoxic pesticides. These neurotoxins have known inhibitory activity against (aceto)cholinesterases.^[4] In early work, representative insecticides were assayed against a single enzyme to identify compounds from observed kinetic rates using the chemometric approach.^[5] Unfortunately, these methods had limited detection to distinguish mixtures of pesticides. A more recent strategy used multiple cholinesterase homologs, establishing the characterization of mixtures of two insecticides using machine learning algorithms.^[5] This technology has since enabled the detection of trace pesticides directly from soil, water, and food samples.^[7]

Our work describes a similar strategy to quantify mixtures of chemically similar native nucleoside and nucleoside analog metabolites, using a set of three dNKs as enzyme-based biosensor. Such therapeutic drug monitoring (TDM) is an established practice within clinical pharmacology and is used to design treatments for optimized efficacy and reduced toxicity.^[8,9] Despite improved clinical outcomes, TDM is currently considered not cost-effective for most treatments including antiretroviral therapy due to the high costs associated with the existing analytical technology.^[10] TDM in patients undergoing nucleoside analog-based therapies is further complicated as a.) treatment routinely uses combinations of two or more therapeutic agents, and b.) nucleoside analogs must be distinguished from native metabolites at physiologically relevant concentrations. Typical blood plasma levels for nucleosides and nucleoside analogs range from 0.5 – 10 μ M.^[11] To evaluate our dNK-based sensor, we selected eight pyrimidine nucleoside as targets for identification and quantification (Fig 1): the two native 2'-deoxynucleosides (thymidine, 2'-deoxycytidine) and six prodrugs of therapeutic relevance. 3'-Azidothymidine (AZT) was the first approved HIV-therapeutic and remains actively prescribed.^[12] 2'-Fluoro-2'-deoxy-1- β -L-arabinofuranosyl-5-methyluracil (L-FMAU) has applications in positron emission tomography imaging.^[13] 2',3'-Didehydro-dideoxy-thymidine (D4T) is used as first-line therapy in resource limited countries.^[12] 2',2'-Difluorodeoxycytidine (gemcitabine, GEM) is standard in cancer care.^[14] 2',3'-Dideoxycytidine (ddC) was amongst the first analogs used in combination drug therapy. Finally, 2',3'-Dideoxy-5-fluoro-3'-thiacytidine (FTC) is a widely prescribed combination antiretroviral agent and is considered standard of care for HIV treatment.^[15]

These nucleoside and nucleoside analogs are phosphorylated to their corresponding monophosphates by different dNKs with characteristic catalytic efficiency. The distinct substrate preference of these enzymes can be exploited for nucleoside detection.^[16] An optimal multisensor contains a highly specific enzyme for each target analyte. Unfortunately, native enzymes rarely fulfill this idealized role. In reality, natural selection provides a variety of homologous enzymes, evolving diverse yet distinct kinetic profiles for the native and

unnatural nucleosides. In this study, three dNKs were selected due to their distinct kinetic profiles for the eight targeted compounds (Fig. 1, Table S1). *Thermotoga maritima* thymidine kinase 1 (TK1) shows exclusive activity for thymidine and its analogs, while human 2'-deoxycytidine kinase (dCK) displays complementary activity for 2'-deoxycytidine and its analogs. Alternatively, *Drosophila melanogaster* deoxynucleoside kinase (dNK) displays broad activity for all investigated substrates. Relying on the distinct activity patterns of these three kinases, data fusion from multiple signals significantly improves analytical consistency and accuracy over a single component system.^[17]

Our approach also distinguishes itself from the previous cholinesterase biosensors by its simpler and more scalable data analysis strategy. Rather than relying on large reference datasets for training of the machine learning algorithm^[7], we measure the time-resolved kinetics of unknown analyte(s) with each dNK, followed by *in silico* comparison of these experimental data with the Michaelis-Menten parameters for the eight reference enzyme-substrate interactions (Fig. 2). More specifically, the computational framework compares uncertainty between observed and expected kinetics, quantified with the Kullback-Leibler divergence.^[18] This uncertainty is translated into a Boltzmann-like ensemble estimating the probability distribution of the unknown sample against the reference substrates. The experimental information from each kinase is then combined using Bayes Theorem, yielding an updated probability for the unknown sample to be a particular reference substrate. Finally, the putative identities are attributed to the most likely substrate(s) that match observed kinetics (for details see supporting information). In regards to scalability, this kinetics-based approach requires only knowledge of Michaelis-Menten parameters for individual substrate-kinase pairs, simplifying the integration of additional nucleosides and nucleoside analogs beyond the current reference substrates.

The concept behind our approach was tested and evaluated in three sets of validation experiments. Initially, we tested the chemometric approach using Michaelis-Menten kinetics to characterize unknown solutions. Eight solutions (A1-A8) were prepared, each containing a single substrate. The individual models for each enzyme (TK1, dCK, dNK) are represented in Fig. 3, depicting the probability distribution across the eight possible substrates. The reactions were performed to completion and the final absorbance quantified the substrate concentration in each sample (Fig. S1). The identity of each substrate was then estimated from comparing the experimental time-course kinetics to expected outputs from known substrate interactions (Table S2). The model robustly distinguished thymidine and deoxycytidine analogs due to the mutually exclusive activities of TK1 and dCK. These two individual enzymes identified the most likely substrate and kinetic data from dNK was largely redundant, affirming the original prediction. The estimated probabilities were then combined in the fusion model shown in Fig. 3, as kinetic information from all three kinases result in higher confidence predictions for the identity of the unknown samples.

In this first series of experiments, the chemometric framework not only identified unknown substrates with >75% probability, but also accurately quantified the total substrate within each sample. With the exception of FTC (A8), all estimated concentrations were within standard deviation of the actual 10 μ M substrate. While the identification of unknown metabolites has limited clinical significance as patients/physicians are usually aware of their

prescribed drugs, this dataset validated the predictive framework. Moreover, the robust and rapid identification of unknown compounds may have direct applications in other disciplines, such as forensic chemistry.

Next, the statistical framework was challenged to analyze two-component mixtures of nucleosides/analogues in an attempt to deconvolute the identity and concentration of each component. The 28 possible combinations of our eight substrates (B1-B28) were prepared, containing 10 – 20 μ M of each component. The systematic mixtures were assayed against the enzyme array (Fig. S2a-c) and analyte identity and concentrations were calculated based on the individual and fused probability distribution profiles (Table S3).

The results of the chemometric model are shown in Fig. 4, describing the probability distribution across all possible combinations. Similar to the previous single component analysis, our method demonstrated the ability to identify and quantify the binary composition of an unknown sample. For 24 of the 28 samples, substrates were identified with >75% probability while four mixtures (B4, B15, B24 and B25) only reached 40-50% probability. In general, samples containing two thymine nucleosides (B1-B3, B8, B9, B14) were the easiest to characterize as TK1 and dNK display opposite preferences for the 3'-modified AZT and unnatural enantiomer L-FMAU. The disparate activity of these two kinases contributes greatly to the overall high probability prediction (>76%) of the fusion model. For these binary mixtures, the predictive framework had nearly identical performance to the previous single component analysis. Similarly, mixtures of a thymine and a cytosine derivative (B4-B7, B10-B13, B15-B22) also inherit generally high confidence (>82%) which can largely be attributed to mutually exclusive recognition of the pyrimidine moiety by TK1 and dCK. Nevertheless, the significant slower catalytic performance parameters of dCK relative to TK1 and dNK creates some ambiguity in fitting the data for B4, B10, B15 and B19. Finally, 2'-deoxycytidine and its derivatives (B23-B28) had the lowest confidence predictions due to the similar observed activity for 3' substituted substrates in both dCK and dNK. This highlights the importance of kinase selection and a potential limitation of enzyme-based characterization. Specifically, samples B24 (dC/ddC) and B25 (dC/FTC) exhibited similar kinetic responses. While the first component (dC) could be attributed with confidence, the second substrate gave an ambiguous characterization between three species (GEM, ddC, and FTC). Similarly, the chemometric model had difficulty quantifying mixtures of similarly reacting species, such as ddC/FTC (B28) and could not algorithmically deconvolute two distinct species. Again, these limitations can largely be attributed to substrate specificity and kinetic parameters of the enzymes used in our study. Replacement of the current deoxynucleoside kinases and inclusion of other native and engineered enzymes with complementary substrate specificities promises an effective strategy to further increase confidence and to broaden the detection capabilities of our chemometric model. In summary, these validation experiments have assessed the ability to deconvolute mixtures, not only distinguishing chemically similar species but also quantifying their abundance. Overall, the data suggests that the chemometric approach can be applied toward analyzing complex mixtures of native nucleosides and their analogs.

To test the impact of a complex sample matrix on biosensor performance, we evaluated our chemometric method with human blood plasma samples spiked with nucleoside analogs.

Specifically, four samples with 1.25, 2.5, 5 or 10 μM of AZT and FTC were prepared, mimicking the TDM of a patient undergoing HIV-treatment. Kinetic time-course measurements with our three kinase sensors (Fig. 5), followed by analysis of the probability distribution profiles were used to verify the identity of the two prodrugs and to estimate their concentrations in the presence of physiological levels of native pyrimidine nucleosides (Table 1).

For all four samples, our chemometric predictive framework correctly quantify the nucleoside analogs added to the plasma samples. Sample quantification was accurate at concentrations above 2.5 μM but noticeably declined at 1.25 μM sample concentration due to the detection limit of the UV-based coupled-enzyme assay.

The detection limit by UV-based assay of $\sim 2 \mu\text{M}$ is approximately one order of magnitude higher than traditional LC-MS approaches (0.1 μM).^[3a] HIV patients undergoing treatment have peak drug concentrations at approximately 10 μM after administration and steadily decline to a target steady-state concentration near 1 μM over the course of hours.^[19] Unfortunately, the lack of resolution at dilute concentrations, coupled with possible background from other biological components, does limit the immediate clinical value of UV-based detection for TDM of antiretroviral therapies.^[20] However, this detection protocol may be suited for TDM of cancer therapies (e.g. gemcitabine), in which steady-state levels are much higher at 20 μM , a more robust detection range.^[21]

To address limited sensitivity of UV-based detection of nucleoside analogs, we also explored the utility of fluorometric detection.^[22] The modified assay offers sensitivity at nanomolar substrate concentrations by coupling nucleoside phosphorylation to production of resorufin. More specifically, formation of pyruvate upon regeneration of ATP by pyruvate kinase is used to produce equimolar quantities of hydrogen peroxide by pyruvate oxidase. In turn, peroxide formation drives conversion of Amplex Red by horseradish peroxidase which can be detected at excitation/emission maxima of 570/583 nm. For proof-of-principle, we used commercially available pyruvate oxidase from acidophilic *Aerococcus* sp. and evaluated the new assay with our single-component samples described earlier (Fig. S3). Fluorometric detection did significantly enhance the sensitivity of the assay, generating a linear spectroscopic signal at nucleoside concentrations as low as 0.1 μM . However, our studies also showed limited utility of the current assay due to overall moderate pyruvate oxidase activity and inhibition in blood plasma. To overcome these limitations, we are considering evaluating oxidases from other organisms or tailoring the enzyme by protein engineering. Separately, concerns over instrument requirements for fluorophor detection have been addressed. We are exploring one possible solution for time-course kinetic measurements via time lapse image acquisition, using a green laser pointer and a cell phone equipped with a 590 nm long-pass filter (Fig. S4).

In summary, we have presented a chemometric method for the enzyme-based detection of multiple nucleosidic analytes using algorithmic analysis. In this initial study, a set of three nucleoside kinases were able to identify and quantify unknown mixtures of up to four different nucleosides and nucleoside analogs from among eight compounds at physiologically relevant concentrations. Beyond the present proof-of-concept, the method is

highly versatile and scalable; novel substrates can be readily added by incorporating their Michaelis-Menten parameters with each kinase sensor. Furthermore, additional kinases can be utilized to increase confidence in the predictive framework and expand the number of detectable substrates including purine nucleosides and their analogs. Finally, the ongoing development and optimization of a fluorometric assay promises a simple and fast detection method for nucleoside and nucleoside analog identity and quantity at physiologically-relevant concentrations.

Experimental Section

Unless otherwise specified, reagents were purchased from Sigma-Aldrich. Stock solutions for nucleosides and nucleoside analogs were prepared in 50 mM Tris-HCl (pH 8) and concentrations determined by UV absorbance. Extinction coefficients used for thymidine and 2'-deoxycytidine: $\epsilon_{260} = 9,000 \text{ M}^{-1} \text{ cm}^{-1}$ and $8,600 \text{ M}^{-1} \text{ cm}^{-1}$, respectively.^[23] For thymine analogs: AZT, L-FMAU (Carbosynth) and D4T: $\epsilon_{260} = 9,700 \text{ M}^{-1} \text{ cm}^{-1}$, $8,800 \text{ M}^{-1} \text{ cm}^{-1}$, and $\epsilon_{280} = 3,200 \text{ M}^{-1} \text{ cm}^{-1}$, respectively. For cytosine analogs: GEM (Eli Lilly), ddC, and FTC: $\epsilon_{260} = 9,400 \text{ M}^{-1} \text{ cm}^{-1}$, $9,100 \text{ M}^{-1} \text{ cm}^{-1}$, and $9,600 \text{ M}^{-1} \text{ cm}^{-1}$, respectively.^[24]

The genes encoding for *T. maritima* thymidine kinase (TK1), *H. sapiens* 2'-deoxycytidine kinase (dCK) and *D. melanogaster* deoxyribonucleoside kinase (dNK) were previously cloned into pET expression vectors (Invitrogen) with IPTG inducible promoters and N-terminal 6xHis tags.^[16, 25] These plasmids were transformed into *E. coli* BL21(DE3) for overexpression followed by protein purification to >90% purity as determined by SDS-PAGE.

Kinase activity was monitored with a spectrophotometric coupled-enzyme assay as described previously.^[26] Briefly, kinase activity was monitored via the regeneration of consumed ATP by pyruvate kinase/lactate dehydrogenase, coupling phosphoryl transfer to the oxidation of NADH. Activity for each substrate was measured by UV absorbance (NADH: $\epsilon_{340} = 6,220 \text{ cm}^{-1} \text{ M}^{-1}$) using a Cary Bio 100 Spectrophotometer (Agilent). Assays were performed at room temperature in 500 μL reaction volume, containing 50 mM Tris-HCl (pH 8), 100 mM KCl, 5 mM MgCl_2 , 1 mM ATP, 0.2 mM phospho-enolpyruvate, 0.2 mM NADH, 5 U of pyruvate kinase (Roche) and 5 U lactate dehydrogenase (Roche). Total enzyme per reaction was constant (1.03 μg TK1, 1.52 μg dCK, 0.727 μg dNK), determined by UV absorbance ($\epsilon_{280} = 10,680 \text{ cm}^{-1} \text{ M}^{-1}$; $56,380 \text{ cm}^{-1} \text{ M}^{-1}$; $39,880 \text{ cm}^{-1} \text{ M}^{-1}$ respectively).^[27] Michaelis-Menten parameters were calculated from triplicate measurements using non-linear regression, and were in agreement with literature data.^[16, 25]

Data analysis from time-resolved kinetic measurements was performed by our predictive model, simulating reactions for established substrate-kinase interactions, statistically comparing expected results to experimental traces, and using a Bayesian model to identify and quantify the most likely substrate(s). The experimental trace (Q_k), described the time-

$$\frac{d[R_{i,k}]}{dt} = \frac{-d[S_i]}{dt} = \frac{K_{cat,i,k} [E_{total,k}] [S_i]}{K_{M,i,k} + [S_i]} \quad (1)$$

dependent (i) substrate distribution assayed against enzyme k . Each reaction was monitored to completion, allowing use of final absorbance change as estimate for initial substrate concentrations $[S_k]_0$ at a known enzyme concentration $[E_{total,k}]$. The expected time-course kinetics ($R_{i,k}$) for enzyme (k) interacting with substrate (i) was estimated using numerical integration of the Michaelis-Menten equation (Equation 1).

The uncertainty between experiment and model was quantified with the Kullback-Leibler divergence ($H_{i,k}$), shown in Equation 2. This uncertainty was translated into a Boltzmann-like ensemble (Equation 3) estimating the probability distribution of the unknown sample against known substrates (Y_j), from an experimental observation (X_j). In this work, $k=3$ (three kinases) and $j=8$ (eight possible substrates) for a substrate of

$$H_{i,k} = \sum_t Q_k(t) \log \left(\frac{Q_k(t)}{R_{i,k}(t)} \right) \quad (2)$$

$$P(X_k|Y_i) = \frac{e^{-H_{i,k}}}{\sum_j e^{-H_{j,k}}} \quad (3)$$

interest (i).

The experimental information from each kinase was then combined, using the joint probability of the unknown sample matching a substrate of interest (Y_j) based on the experimental kinetic traces from all three kinases (X_k), shown in Equation 4. The statistical model, derived from Bayes Theorem, assumed conditional independence, stating the observed

$$P(Y_i|X_1, \dots, X_k) = \frac{P(Y_k) \prod_i P(X_k|Y_i)}{\sum_j P(Y_j) \prod_i P(X_k|Y_j)} \quad (4)$$

activity was not correlated among kinases.

The higher order mixtures had a similar mathematical framework; however, the expected substrate distributions were described as a linear sum of Michaelis-Menten reactions. A modification was that initial concentrations of mixtures were estimated from nonlinear regression to the multiple reaction rates for each substrate. In most cases, two or more distinct kinetic rates were observed (i.e. 'slow' and 'fast' reactions), which was deconvoluted to estimate concentrations for the distinct substrates. If two substrate concentrations could not be determined from the kinetic data, the algorithm assumed an equimolar mixture. The identities were attributed using a similar Bayesian framework to return the most likely combination of substrates that match the observed kinetics.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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thymidine	AZT	L-FMAU	D4T									
TK1	dCK	dNK	TK1	dCK	dNK	TK1	dCK	dNK	TK1	dCK	dNK	
k_{cat}/K_M ($\text{mM}^{-1} \text{s}^{-1}$)	400	nd	14,000	380	nd	55	45	nd	6,100	15	nd	9

2'-deoxycytidine	GEM	ddC	FTC									
TK1	dCK	dNK	TK1	dCK	dNK	TK1	dCK	dNK	TK1	dCK	dNK	
k_{cat}/K_M ($\text{mM}^{-1} \text{s}^{-1}$)	nd	25	10,000	nd	11	12,000	nd	6	230	nd	2	110

Figure 1.

Summary of selected pyrimidine nucleoside and nucleoside analogs and their catalytic performance (k_{cat}/K_M) for *Thermotoga maritima* thymidine kinase 1 (TK1), human 2'-deoxycytidine kinase (dCK) and *Drosophila melanogaster* 2'-deoxyribonucleoside kinase (dNK).

Figure 2.

General method to compare time-resolved kinetic measurements of unknown analytes to expected outputs of reference substrates based on their Michaelis-Menten kinetics. The reaction time-course of TK1 with an unknown sample, as well as expected reaction profiles for four reference substrates (thymidine, AZT, L-FMAU, and D4T) are shown. The difference between the expected and observed distributions for each unknown-reference pair allows for sample identification via its probability distribution profile.

Figure 3.

Probability distribution of single-component samples. Each column represents a sample of A1-A8 assayed against the indicated kinase. Each row represents a statistical comparison from the expected kinetic output of the indicated kinase with the experimental sample. The grey scale qualitatively describes the probability of each candidate model while the blue circles mark the actual sample identity. The fusion model combines kinetic information from each kinase, returning the most likely substrate present in each duplicate sample.

Figure 4.

Probability distribution for binary component mixtures. Each column represents a sample (B1-B28) assayed against the representative kinases. Each row corresponds to the probability an analyte is detected in a given sample, with the grey scale indicative of the assigned confidence. Blue circles mark the actual sample identity. The component models for each kinase and combined probabilities as indicated.

Figure 5.

Time-resolved kinetic measurements of blood plasma samples spiked with four different concentrations of AZT and FTC using TK1, dCK and dNK. The traces were analyzed with our predictive framework to identify and quantify the nucleoside analogs in the presence of physiological concentrations of 2'-deoxycytidine and thymidine.

Table 1

Calculated composition of spiked blood plasma samples.

Analyte	1.25 μ M AZT/FTC	2.5 μ M AZT/FTC	5 μ M AZT/FTC	10 μ M AZT/FTC
[FTC]	1.9 $\pm$ 0.5	3.2 $\pm$ 0.5	5.7 $\pm$ 0.6	10.2 $\pm$ 1.0
[AZT]	1.1 $\pm$ 0.3	2.5 $\pm$ 0.4	5.5 $\pm$ 0.6	10.7 $\pm$ 1.1
2'-dC	1.7 $\pm$ 0.5	1.6 $\pm$ 0.2	1.4 $\pm$ 0.1	1.0 $\pm$ 0.1
Thym	0.9 $\pm$ 0.2	0.9 $\pm$ 0.1	1.2 $\pm$ 0.1	1.5 $\pm$ 0.2

All experiments performed in triplicates.