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Monitoring of phosphorylation using immobilized kinases by on-line enzyme bioreactors hyphenated with High-Resolution Mass Spectrometry

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Abstract

Nucleosides analogues are the cornerstone of the treatment of several human diseases. They are especially at the forefront of antiviral therapy. Their therapeutic efficiency depends on their capacity to be converted to the active nucleoside triphosphate form through successive phosphorylation steps catalyzed by nucleoside/nucleotide kinases. In this context, it is mandatory to develop a rapid, reliable and sensitive enzyme activity test to evaluate their metabolic pathways. In this study, we report a proof of concept to directly monitor on-line nucleotide multiple phosphorylation. The methodology was developed by on-line enzyme bioreactors hyphenated with High-Resolution Mass Spectrometry detection. Human Thymidylate Kinase (hTMPK) and human Nucleoside Diphosphate Kinase (hNDPK) were covalently immobilized on functionalized silica beads, and packed into micro-bioreactors (40

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μL). By continuous infusion of substrate into the bioreactors, the conversion of thymidine monophosphate (dTMP) into its di- (dTDP) and tri-phosphorylated (dTTP) forms was visualized by monitoring their Extracted Ion Chromatogram (EIC) of their $[M-H]^-$ ions. Both bioreactors were found to be robust and durable over 60 days (storage at 4°C in ammonium acetate buffer), after 20 uses and more than 750 minutes of reaction, making them suitable for routine analysis. Each on-line conversion step was shown rapid (< 5 min), efficient (conversion efficiency > 55 %), precise and repeatable (CV < 3% for run-to-run analysis). The feasibility of the on-line multi-step conversion from dTMP to dTTP was also proved. In the context of selective antiviral therapy, this proof of concept was then applied to the monitoring of specificity of conversion of two synthesized Acyclic Nucleosides Phosphonates (ANPs), regarding human Thymidylate Kinase (hTMPK) and *vaccina virus* Thymidylate Kinase (*vv*TMPK).

Keywords: Drug discovery, high resolution mass spectrometry, immobilized kinases, on-line enzymatic phosphorylation.

1. Introduction

Drug discovery is a lengthy process. To gain a better understanding of the activity and toxicity of pharmaceutical drugs, it is important to determine their metabolic pathways [1, 2]. The monitoring of conversion efficiency helps to optimize the drug development process. One illustration of this latter is the activation of nucleotide analogues into their triphosphate active form [3-5]. These compounds are the cornerstone of the treatment of several human diseases and are especially at the forefront of antiviral therapy [6-9]. Biological activities are exhibited by triphosphate nucleotides, which compete with natural substrates for incorporation in the newly synthesized DNA strand, thus causing the inhibition of viral polymerase activity (e.g.

DNA chain termination) and therefore virus replication [10, 11]. To ensure good stability and high availability, these compounds are delivered as uncharged molecules [12]. Two major antiviral nucleoside classes have proved to be biologically active: the nucleoside analogues [13] and the Acyclic Nucleoside Phosphonate (ANPs) analogues [7]. Since the rate-limiting step of the drug activation is the conversion of nucleoside analogues to their monophosphate form, nucleotide analogues as ANPs were designed to circumvent the initial phosphorylation activation step [14]. The phosphorylation process of a monophosphate to its triphosphate counterpart involves two successive enzymatic phosphorylation reactions catalyzed by cellular kinases [14, 15]. For instance, the conversion of the endogenous thymidine monophosphate (dTMP) to thymidine triphosphate (dTTP) is catalyzed by the human Thymidylate Kinase (hTMPK) and the human Nucleoside Diphosphate Kinase (hNDPK) [11]. This activation is performed by a phosphotransfer reaction from a donor, usually the γ-phosphate of adenosine-5'-triphosphate (ATP), towards an acceptor such as the 5'-OH nucleoside or α-, β-phosphate nucleotide groups, in the presence of a chelating agent (usually Mg^{2+}) [3-5, 16]. To the best of our knowledge, the main techniques used to determine the efficiency of a kinase catalyzed reaction are spectrophotometric [17-19], radioisotopic [20-22], High Performance Liquid Chromatography (HPLC) [23] and Capillary Electrophoresis (CE) [24-26]. Although all these techniques have shown their potential for the study of kinase activity, the development of an on-line mass spectrometric approach makes sense in view of its rapidity, substrate versatility and specificity of detection. These on-line methodologies are currently gaining interest for application in drug discovery [27-32]. This liquid chromatography methodology uses biological agents as stationary phase to study enzyme/ligand interactions [33]. Enzyme-immobilized bioreactors offer usually various advantages such as purification of the biological environment (low matrix effect), preservation of activity, bioreactor stability (possible reuse), ease of analysis, easy coupling

with the detection system, direct conversion monitoring and possible high throughput screening of compound mixtures. Recently, the use of on-line methodologies with a single bioreactor or multi-reactor have been presented in the field of substrate conversion monitoring [34, 35] or DNA digestion [36].

In this context, the present study aimed to develop a rapid, efficient and versatile on-line methodology for the direct monitoring of nucleoside monophosphate and nucleotide diphosphate phosphorylation. As a proof of concept, individual conversion of dTMP into dTDP and dTDP into dTTP were monitored by on-line infusion into immobilized hTMPK and hNDPK bioreactors. The feasibility of the on-line two successive phosphorylation steps was also evaluated. This on-line system showed various advantages such as a rapid (less than 5 minutes) and efficient (more than 50%) conversion. Moreover, thanks to the dual loop system, this system is versatile (successive substrate infusion) and automatable. It also allows to directly visualize the drug conversion with the specific detection by mass spectrometry. This developed on-line methodology was then applied to the qualitative study of specificity of conversion of two chemically synthesized Acyclic Nucleotides Phosphonates (ANPs) named ANP-CH_3 and ANP-Br [37] (molecular formula shown in Figure 1) regarding two TMPKs: *vaccina virus* TMPK (*vv*TMPK) and human TMPK (hTMPK).

2. Experimental section

2.1. Material

Thymidine 5'-monophosphate disodium salt hydrate (dTMP), thymidine 5'-diphosphate sodium salt (dTDP), thymidine 5'-triphosphate sodium salt (dTTP), adenosine 5'-triphosphate magnesium salt (ATP), ammonium acetate ($AcNH_4$) and magnesium chloride ($MgCl_2$) were purchased from Sigma-Aldrich (Saint-Quentin-Fallavier, France). For each experiment, $AcNH_4$ was used at 50 mM ionic strength at pH = 7. The bioreactor (50 x 1 mm stainless steel

capillary), column end fitting, high pressure unions, back pressure regulator (500 psi) and PEEK tubing (1 mm and 0.75 mm) were purchased from Cluzeau Info Labo (Sainte-Foy-La-Grande, France). The de-ionized water used in all procedures was purified with an Elgastat UHQ II system (Elga, Antony, France). Plasmids bearing hTMPK, *vv*TMPK and recombinant hNDPK were a generous gift from the University Pierre et Marie Curie (UPMC-Paris 6). Recombinant hTMPK was produced and purified as previously described [38]. Acyclic nucleoside monophosphates ANP-CH_3-MP and ANP-Br-MP were synthesized as described in our previous work [37].

2.2. Instrumentation

Experiments were performed on a Bruker maXis UHR-Q-TOF spectrometer (Bremen, Germany) in negative ion mode. The mass spectrometer was coupled on-line with a Dionex UltiMate 3000 RSLC system (Germering, Germany). Before each experiment, calibration was performed using ES-TOF tuning mix (Agilent Technologies, Les Ulis, France). The capillary voltage was set at - 4000 V, the nebulizer pressure at 0.6 bar, the dry gas flow at 7.0 L min^{-1} and heated at 200°C. Spectra were recorded within the mass range from *m/z* 50 to 1350. Chromatograms of the ions of interest (*m/z* 321.0500 $[M-H]^-$ for dTMP, *m/z* 401.0166 $[M-H]^-$ for dTDP and *m/z* 480.9831 $[M-H]^-$ for dTTP, *m/z* 259.0562 $[M-H]^-$ for ANP-CH_3-MP, *m/z* 322.0561 $[M-H]^-$ for ANP-Br-MP, *m/z* 339.0150 $[M-H]^-$ for ANP-CH_3-DP and *m/z* 402.0149 $[M-H]^-$ for ANP-Br-DP) were generated as Extracted Ion Chromatograms (EIC) within a ± 0.005 *m/z* range. *m/z* values are summarized in Table 1. All data were analyzed using DataAnalysis 4.4 software (Bruker).

2.3. Enzyme immobilization

hTMPK, *vv*TMPK and hNDPK immobilizations were performed following a previous study [39]. Briefly, separate immobilizations *via* glutaraldehyde cross-linking agent were performed

by placing the enzyme in contact with 30 mg of silica beads diluted in phosphate buffer (500 µL, 50 mM ionic strength, pH = 7) with final excess concentrations of 0.55 mg mL^{-1} of hTMPK and *vv*TMPK and 1.8 mg mL^{-1} of hNDPK. The three solutions were stirred for 6 h at 4°C and then stored overnight with 1 mM glycine at 4°C. Remaining free hTMPK, *vv*TMPK and hNDPK were removed by successive washings ($AcNH_4$).

2.4. Bioreactor packing

Each immobilized enzyme solution was placed in a tank (100 x 4.6 mm) connected to the bioreactor (50 x 1 mm) by means of a PEEK tube of 1 mm id and a true zero dead volume union. A frit was placed at the end of the bioreactor to retain the silica beads. $AcNH_4$ buffer was eluted through the tank with a semi-micro HPLC pump (PU-2085 Plus, Jasco, Tokyo, Japan). Start flow was set at 10 µL min^{-1} and was gradually increased to reach a maximum pressure of 30 bar. After 1 h of elution, a back-pressure regulator was added at the end of the bioreactor. At the maximum pressure of 40 bar, silica was considered packed into the bioreactors. The bioreactor denaturation was performed by overnight MeOH infusion at 60 µL min^{-1}.

2.5. On-line fluidic system

As shown in Figure 2, the on-line system consisted in a high flow rate precision pump and a ten-port valve containing 2 injection loops connected to the bioreactors (active and denatured). $AcNH_4$ buffer was eluted at 40 µL min^{-1} (or 60 µL min^{-1}) through the ten-port valve. On this valve, two 320 µL injection loops, build with 0.75 mm id PEEK tubes were placed in back flush configuration (Figure S1). Injections were made at position 7 by means of an autosampler. The autosampler injected 400 µL of a solution "1" into the loop 1 (position valve 1-2) with the loading pump. The valve then switches to position 1-10 to infuse solution "1" into the bioreactors with the analytical pump ($AcNH_4$ buffer). During loop 2 infusion, the

loop 1 is washed with AcNH4 buffer and a solution "2" is loaded into the loop 2 with the loading pump (position valve 1-10). When the infusion is completed, the valve switches to position 1-2 and loop 2 is infused. Solutions "1" and "2" can be the same for a longer time infusion or can be different for a screening approach. Each parameter (time infusion, injection, valve position) is programmed manually in the method (script editor). The valve switched every 3 or 5 minutes (for flow rates of 60 µL min^{-1} and 40 µL min^{-1}, respectively). The two bioreactors, joined in series at 37°C in an oven, are connected to the ten-port valve at position 4 from the inlet and directly linked to the electrospray source from the outlet.

2.6. Validation of the on-line methodology for individual or multi-step enzymatic reaction

2.6.1. hTMPK bioreactor conversion

The bioreactor was conditioned at 37°C by $AcNH_4$ buffer elution for 15 minutes at a flow rate of 60 µL min^{-1}. 400 µL of a 20 µM dTMP solution ($AcNH_4$, [ATP] = 150 µM, [$MgCl_2$] = 200 µM,) were injected *via* the loop system as previously described. After the valve switch, the sample contained in the loop was eluted with $AcNH_4$ at a flow rate of 60 µL min^{-1} (P = 10 bar) into the hTMPK bioreactor. During the first loop infusion, 400 µL of the same dTMP solution were injected into the second loop. After 3 minutes of infusion, the valve was switched to infuse the second loop. This process was repeated until the EIC plateau for dTMP and dTDP was reached. The bioreactor was then washed and the enzymes were regenerated with $AcNH_4$ at 100 µL min^{-1} until elimination of the substrate and product from the capillary. Between each use, the hTMPK bioreactor was stored at 4°C in $AcNH_4$. The same methodology was applied to the *vv*TMPK bioreactor study.

2.6.2. hNDPK bioreactor conversion

176 The hNDPK bioreactor was conditioned as previously described. The dTDP solution (20 µM
177 in $AcNH_4$, [ATP] = 150 µM, [$MgCl_2$] = 200 µM) was continuously infused into the hNDPK
178 bioreactor at a flow rate of 40 µL min^{-1} (5 minutes between each valve switch). Analyses
179 were performed after dTDP and dTTP EIC plateau monitoring. The bioreactor was then
180 washed and the enzymes were regenerated with $AcNH_4$ at 100 µL min^{-1} until elimination of
181 the substrate and product. Between each use, the hNDPK bioreactor was stored at 4°C in
182 $AcNH_4$.

183 **2.6.3. Multi-step phosphorylation with hTMPK and hNDPK bioreactors in**
184 **series**

185 The bioreactors were placed in series *via* a stainless steel high-pressure union. 20 µM dTMP
186 solution ([ATP] = 150 µM, [$MgCl_2$] = 200 µM) was infused at 40 µL min^{-1} (P = 11 bar) as
187 previously described (5 minutes between each valve switch). Analysis was stopped after
188 monitoring the dTMP, dTDP and dTDP EIC plateaus. The bioreactors were washed and the
189 enzymes were regenerated with $ACNH_4$ elution at 80 µL min^{-1}.

190 **2.6.4. Stability and reuse of the hTMPK and hNDPK bioreactors study**

191 h*TMPK bioreactor:* 20 µM dTMP solution ([ATP] = 150 µM, [$MgCl_2$] = 200 µM) was
192 infused at 60 µL min^{-1} with $AcNH_4$ in the hTMPK bioreactor. Seven points of activity were
193 recorded over 60 days. Over this period, the bioreactor was used 21 times, corresponding to
194 795 min of use. The hTMPK bioreactor was alternatively used and stored at 4°C in $AcNH_4$
195 for 60 days.

196 h*NDPK bioreactor:* 20 µM dTDP solution ([ATP] = 150 µM, [$MgCl_2$] = 200 µM) was
197 infused at 40 µL min^{-1} with $AcNH_4$ in the hNDPK bioreactor. Seven points of activity were
198 recorded over 68 days (20 uses, corresponding time of use 915 minutes). The hNDPK
199 bioreactor was alternatively used and stored in ammonium acetate buffer at 4°C for 68 days.

2.7. Application of the on-line methodology for the study of two ANPs specific conversion regarding hTMPK and *vv*TMPK

hTMPK and *vv*TMPK bioreactors were individually placed in the fluidic system as described **in paragraph 2.6.1.** The bioreactors were conditioned at 37°C with $AcNH_4$ buffer elution for 15 min at a flow rate of 60 µL min^{-1}. 400 µL of 20 µM of each substrate solution (dTMP, ANP-CH_3-MP and ANP-Br-MP in $AcNH_4$, [ATP] = 150 µM and [$MgCl_2$] = 200 µM,) were injected via the loop system as previously described. Substrate solutions were continuously infused until the detection of EIC plateau. Between each substrate infusion, the bioreactors were washed and the enzymes were regenerated with $AcNH_4$ at 100 µL min^{-1} until elimination of substrate and product from the capillary.

3. Results and discussion

3.1. Monitoring phosphorylation – Optimization of the on-line methodology conditions

In this study, optimizations of the system were conducted. The phosphodonor and chelatant concentrations were evaluated for the conversion efficiency and the fluidic system and the bioreactor packing (Figure S2) were optimized. The influence of [ATP] and [$MgCl_2$] on ionization and on the mono-phosphorylation step was evaluated in our previous study with the same enzymes [39]. The optimal concentration of the phosphodonor (ATP) for on-line conversion was evaluated for the first reaction step, using the hTMPK bioreactor. Based on the results shown in Figure S3**,** the optimum concentration was fixed at 150 µM of ATP, in terms of conversion rate, conversion speed and detector saturation. This concentration was then fixed for the study of the hNDPK bioreactor.

The immobilization step was carried out with high enzymes concentrations in order to saturate the immobilization sites as described in our previous study [39]. Free and immobilized

224 enzyme activities were evaluated by FIA-HRMS. The immobilized enzyme activities were
225 evaluated by Michaelis-Menten study by Flow Injection Analysis hyphenated with High
226 Resolution Mass Spectrometry, as described in our last work [39]. Briefly, similar K_M values
227 for immobilized and free enzymes were obtained regarding dTMP [$K_{M(\text{free hTMPK})}$ = 34.43 ±
228 5.05 µM, $K_{M(\text{immobilized hTMPK})}$ = 27.92 ± 4.14 µM and $K_{M(\text{free hNDPK})}$ = 92.75 ± 12.80 µM,
229 $K_{M(\text{immobilized hNDPK})}$ = 132.90 ± 18.50 µM], showing that the immobilization step does not
230 influence the enzyme's affinity towards its substrate. The reaction speed (V_{max}) of the
231 immobilized enzymes was reduced by half [$V_{max(\text{free hTMPK})}$ = 6.86 ± 0.51 µM min^{-1},
232 $V_{max(\text{immobilized hTMPK})}$ = 2.94 ± 0.17 µM min^{-1} and $V_{max(\text{free hNDPK})}$ = 13.01 ± 1.27 µM min^{-1},
233 $V_{max(\text{immobilized hNDPK})}$ = 6.41 ± 0.36 µM min^{-1}]. Immobilized hTMPK and hNDPK activities
234 were proved to be sufficient to evaluate on-line phosphorylation reactions. In this context,
235 these enzymes were used for the on-line reactions studies.

236 As shown in Figure 2, the system consists of a dual injection loop (2 loops of 320 µL)
237 connected to a ten-port valve. The volume of the two loops, arbitrarily fixed at 320 µL to
238 obtain an elution time of 5 min for each one (flow rate fixed at 50 µL min^{-1}), is adjustable
239 depending on the elution time required. This system provides a constant ligand infusion into
240 the bioreactors with high versatility (no mobile phase changes or lengthy reconditioning
241 between ligand analyses) and without LC system salt contamination. Ligand injection into the
242 loop can be performed manually by means of a syringe (for method development) or by
243 programming automatic injection *via* the use of a second pump and an autosampler. This
244 automation makes the system compatible for routine analysis and for molecule screening.
245 Each bioreactor was evaluated for one-step reaction in terms of feasibility, conversion rates,
246 stability and analysis repeatability. For both bioreactors, each specific substrate (dTMP and
247 dTDP) was infused into active and denatured hTMPK and hNDPK bioreactors respectively, to
248 estimate false positive conversion detection and to evaluate non-specific interactions. The

feasibility of the on-line two successive phosphorylations was then evaluated before the application study.

3.2. Validation of the on-line approach for single step phosphorylation monitoring

3.2.1. hTMPK bioreactor

The conversion of nucleoside monophosphate into nucleoside diphosphate is known to be the reaction-limiting step [10, 14]. hTMPK bioreactor conversion was evaluated by natural substrate (dTMP) infusion. The reaction was directly checked by monitoring the specific EIC of the masses of interest. During the infusion step (60 µL min^{-1}) the substrate first bound to the enzyme. After full saturation of the active sites, dTMP passed through the bioreactor and the EIC related to the non-retained substrate appeared. The product was then released from the bioreactor and the dTDP EIC was delayed. Figure 3 shows the EIC of dTMP and dTDP during the infusion of dTMP solution into the denatured and active bioreactor. No conversion was achieved with the denatured hTMPK bioreactor, attesting the lack of a false positive. In the case of active hTMPK, dTDP appeared rapidly, after 2 min of infusion (30 s after dTMP accumulation). The plateau conversion was quickly reached after 4 min of dTMP infusion. Compared to the experiment with denatured enzyme, a decrease in dTMP intensity was observed (from 3.8 x 10^5 a.u for denatured to 1.2 x 10^5 a.u for active hTMPK) and the plateau was delayed (from 4 to 8 minutes), which confirmed the substrate consumption. The remaining dTMP EIC intensity was not directly correlated to the non-conversion of dTMP into dTDP but it was also due to in-source dTDP fragmentation. This fragmentation could be estimated at 20% by analyzing a range of dTDP concentrations (5-50 µM). The on-line conversion efficiency of dTMP into dTDP was therefore estimated at 67%. Although a decrease in dTMP intensity can be noted (4.3 min, active hTMPK), the feasibility and the non-interference of the switch loop system during valve rotation is proved. This experiment

attested both the feasibility of an on-line phosphorylation and the ability to ensure constant substrate infusion *via* the use of the dual switch loop system developed for this study.

3.2.2. hNDPK bioreactor

To validate the versatility of this method, the hNDPK bioreactor activity was checked by dTDP infusion. As shown in Figure 4, similar results were obtained. The absence of conversion, using denatured hNDPK, attested the lack of false-positive whereas product accumulation (dTTP) was observed with the active bioreactor. Total conversion of dTDP into dTTP was observed. The remaining intensity of dTDP EIC is due to the estimated 50% of dTTP fragmentation. The full conversion is due to the higher V_{max} value of immobilized hNDPK ($V_{max(immobilizd\ hNPK)} = 6.4\ \mu L\ min^{-1}$ and $V_{max(immobilized\ hTMPK)} = 2.9\ \mu L\ min^{-1}$) that enable a faster conversion. This maximal velocity has an even more important influence on the conversion rate in on-line enzymatic conversion. These experiments proved the feasibility to efficiently (conversion estimation > 60%) and rapidly (< 5 minutes) monitor individual on-line phosphorylation.

3.3. Validation of the on-line approach for multi-step phosphorylation monitoring

On-line individual reactions were shown to be feasible and efficient by the present methodology. The feasibility of the direct monitoring of dTTP accumulation by the on-line multi-step enzymatic reaction was studied, as a proof of concept, by placing the two bioreactors in series in the LC system. The conversion of dTMP into its di- and tri-phosphate form was directly monitored by EIC. Similar conditions to those previously described were applied. Figure 5 shows the EIC of the masses of interest. The initial substrate (dTMP) appeared after 5 min of infusion whereas the accumulation of the intermediate (dTDP) and final products (dTTP) appeared at 10 minutes, reaching a plateau at around 25 minutes. Conversion was shown to be efficient with 55% of dTMP converted into dTTP. The high

297 intensity of dTMP EIC is due to the limiting step of dTMP conversion into dTDP but also due
298 to the fragmentation of dTDP and dTTP as explained above. The cascade efficiency was
299 consistent with individual reaction efficiencies (55% conversion of dTMP into dTTP for the
300 cascade against 67% and 100 % for each individual bioreactor).

301 **3.4. Bioreactor repeatability and reuse study**

302 The system repeatability and stability were further evaluated. The repeatability of hTMPK
303 and hNDPK bioreactor activities was studied through three successive infusions (n = 3) of
304 dTMP (hTMPK bioreactor) and dTDP (hNDPK bioreactor). Relative standard deviations for
305 run-to-run analysis were acceptable with 2.42% for the hTMPK bioreactor and 2.36 % for the
306 hNDPK bioreactor. Longevity was investigated by monitoring bioreactor activities over
307 several days. Figure 6 shows a decrease in normalized bioreactor activities in function of
308 time. The hTMPK bioreactor was still active after 795 minutes, 21 uses and 60 days of
309 storage with a normalized remaining activity of 10%. The hNDPK bioreactor showed better
310 stability with a loss of 60% of the initial activity after 965 minutes, 20 uses and 68 days of
311 storage. The bioreactors can therefore be regenerated after each analysis and can be used for
312 multiple analyses performed over several days. Therefore, a limitation of this methodology
313 could turn out to be the decrease of bioreactor stability over the time. This limitation could be
314 easily overcome with the injection of a reference compound (such as dTMP).

315 **3.5. Application of the on-line methodology: study of ANPs specific conversion**
316 **regarding hTMPK and *vv*TMPK**

317 ANPs have become a key class of antiviral nucleotide derivatives. Following their uptake,
318 ANPs undergo most often two phosphorylation steps catalyzed by cellular kinases to reach
319 their target, the viral DNA polymerase [11]. Concerning the ANPs study, the first
320 phosphorylation step (from MP to DP) is most often described as the rate-limiting step

(compared to DP-TP conversion) [10]. In this context, the developed on-line methodology was applied to the study of the mono-phosphorylation step. The selectivity of antiviral therapy is an important aspect in drug discovery and could have an important impact on pharmacological and toxicological outcomes. One way to design site-selective therapy is the use of viral kinases to convert pro-drug into active nucleoside triphosphate locally in the infected cells. In this work, the feasibility of the on-line monitoring phosphorylation was evaluated for the study of conversion specificity of two ANPs phosphorylations (Figure 1). The ANPs containing a pyrimidine base were proven efficiently activated by viral and human TMPK. The influence of the aliphatic chain length (3, 4 and 5 carbones) and the kind of C_5 substituent was evaluated. hTMPK was shown to efficiently phosphorylate (E)-isomer of allylphosphonate uracil containing a methyl (ANP-CH_3) or a bromine (ANP-Br) at C_5-position whereas *vv*TMPK exhibited reduced activity towards both substrates [37]. hTMPK and *vv*TMPK activities regarding ANP-CH_3-MP and ANP-Br-MP were first evaluated by UV-spectrophotometry assay and compared with activities toward the endogenous substrate dTMP. Results are shown in supplementary data Table S1. hTMPK showed high activity regarding the two ANPs with $k_{cat}/K_{M\ (ANP\text{-}CH3\text{-}MP)}$ = 25 000 M^{-1} s^{-1} and $k_{cat}/K_{M\ (ANP\text{-}Br\text{-}MP)}$ = 9 000 M^{-1} s^{-1} (respectively 6 and 17 times lower than hTMPK activity regarding dTMP). *vv*TMPK showed lower activity regarding the same substrates with $k_{cat}/K_{M\ (ANP\text{-}CH3\text{-}MP)}$ = 1 380 M^{-1} s^{-1} and $k_{cat}/K_{M\ (ANP\text{-}Br\text{-}MP)}$ = 1 200 M^{-1} s^{-1} (respectively 72 and 83 times lower than *vv*TMPK activity regarding dTMP).

The proof of concept was developed with the study of the endogenous compound dTMP regarding two human kinase strains TMPK and NDPK. The study proved the feasibility of the on-line method to directly monitor on-line phosphorylation. Two ANPs were then evaluated to study the possible transposition of the method to other substrates (synthesized, with lower

345 conversion rate) regarding other kinase strains (*vaccina virus*), in order to show the potential
346 of the methodology to realize a screening of various compounds.

347 The feasibility of the on-line conversions was evaluated by direct infusion of dTMP, ANP-
348 CH_3-MP and ANP-Br-MP into immobilized hTMPK and *vv*TMPK bioreactors. As *vv*TMPK
349 shares similar structure with hTMPK [40] the two immobilization processes and efficiencies
350 were considered similar. The monitoring of the specific EIC of masses of interest offered a
351 direct monitoring of the reactions. *vv*TMPK activity was first validated by a
352 spectrophotometric assay. Results are shown in Table S1.

353 Figure 7 shows the EIC of interest during dTMP, ANP-CH_3-MP and ANP-Br-MP infusion
354 into active and denatured hTMPK bioreactor. Denatured hTMPK bioreactor gave no
355 conversion for the three substrates which attest to the absence of false positive. As expected
356 with our previous analysis, dTMP conversion was confirmed by infusion into active hTMPK
357 bioreactor. The on-line analyses of ANPs highlighted an accumulation of diphosphate form
358 after 15 min of infusion. Although the catalytic efficiencies of hTMPK regarding ANP-CH_3-
359 MP and ANP-Br-MP respectively were found lower than the catalytic efficiency of dTMP
360 conversion (Table S1), the on-line experiments and immobilized enzymes showed a good
361 correlation with a specific and significant conversion monitoring. Unlike natural substrate, the
362 diphosphate ANP standards are not commercially available. In addition, their ionization
363 disparity and the EIC intensity of diphosphate ANPs cannot be compared to evaluate reaction
364 efficiencies. Although, in this application, this approach is not quantitative, the latter offers
365 various attractive advantages such as a speed of analysis (4 min for dTMP and 15 min of
366 ANPs), a specificity of detection (without a chromatographic dimension), a versatility of
367 analysis (same analytical method for various synthetic compounds) and an identification of
368 the reaction product (HRMS detection) for a better knowledge of phosphorylation mechanism
369 of each drug. To confirm the effectiveness of this methodology, the same ANPs were

analyzed with the *vv*TMPK bioreactor. As shown in Figure 8, *vv*TMPK bioreactor activity was first validated by dTMP infusion. The on-line experiments seem to be correlated with the catalytic efficiency of free *vv*TMPK ($k_{cat}/K_{M(vvTMPK)}$ = 100 000 M^{-1} s^{-1}. Indeed, a significant conversion of dTMP in dTDP was observed. In the case of *vv*TMPK, the dTMP conversion was shown lower. In addition to a lower catalytic efficiency of *vv*TMPK, this lower conversion is likely due to the storage time of the enzyme (hTMPK freshly produced).

In case of ANPs infusion into *vv*TMPK bioreactor, the results showed no conversion that attest to the lack of false positive. These results are consistent with the results obtained with the spectrophotometric test (Table S1). In order to improve the confidence of this experiments, the off-line FIA-HRMS methodology was also performed to validate this specificity of conversion. As shown in Figure S5, the on-line monitoring results are also consistent with the off-line FIA-HRMS methodology. These two experiments showed the feasibility of evaluating the activity specificity of the human and *vaccina virus* enzymatic strains regarding two ANP compounds synthesized in the laboratory. These results validated the absence of false positive and/or false negative thus validating the reliability of the on-line method. Indeed, the results obtained with the FIA-HRMS method (off-line sample preparation, time-consuming) with overnight reaction were shown similar to those obtained with the on-line methodology within 15 minutes. This on-line methodology was proved reliable, rapid, automatable and therefore suitable for a screening methodology.

4. Conclusion

This work shows for the first time the feasibility of an on-line MS methodology for the conversion monitoring of nucleoside monophosphate (NMP) into nucleoside diphosphate (NDP) and triphosphate (NTP). As a proof of concept, the two-phosphorylation steps from thymidine monophosphate to thymidine triphosphate were monitored by the methodology.

This dynamic approach showed various attractive advantages such as specificity of detection, speed of analysis (5 minutes), high conversion efficiency (> 50 %), substrate versatility (no chromatographic dimension) and possible automation (for screening methodology). In this context, this work offered an example of application of the on-line methodology for the monitoring of conversion of two Acyclic Nucleoside phosphonates (ANPs) regarding human and *vaccina virus* TMPK strains. This approach was proven reliable regarding the specificity of conversion and results obtained were in agreement with the spectrophotometric and the FIA-HRMS assays. Therefore, thanks to the longevity of bioreactor, the possible automation and multi-step enzymatic monitoring, this methodology could be easily applied to the metabolization study of various new potential antiviral drugs, whether for one step or for multi-step phosphorylation

Although this work was developed by High-Resolution Mass Spectrometry, this methodology is transposable to less expensive mass spectrometers (MRM mode). Actually, this development can be applied to the study of other synthetic (pro)drugs in order to bring better comprehension of a potential drug metabolic pathway: phosphorylation steps, specific activity regarding human or viral enzymatic strains….

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Figure captions

Figure 1: Chemical formula of (a) Acyclic Nucleoside Phosphonate-CH3 (ANP-CH_3-MP) and (b) Acyclic Nucleoside Phosphonate-Br (ANP-Br-MP).

Figure 2: On-line experiment methodology; 2 sample loops of 320 µL connected to the two bioreactors in series via a 10-port valve. The bioreactors are directly connected to the high-resolution mass spectrometer via an electrospray source. The autosampler injects a solution "1" in the loop 1 (position valve 1-2) with the loading pump. The valve switches to position 1-10 to infuse solution "1" into the bioreactors with the analytical pump. During solution "1" infusion, a solution "2" is loaded into the loop 2 with the loading pump (position valve 1-10). When the infusion is completed (time infusion programmed in the method), the valve switches to position 1-2 and loop 2 is infused with the analytical pump.

583 **Figure 3:** hTMPK bioreactor; monitoring of EIC for dTMP and dTDP (m/z 321.0500 $[M-H]^-$ for
584 dTMP, m/z 401.0166 $[M-H]^-$ for dTDP) for (a) denatured and (b) active bioreactors. [dTMP] = 20 µM
585 ([ATP] = 150 µM, $[MgCl_2]$ = 200 µM), flow rate = 60 µL min^{-1}, mobile phase: ammonium acetate
586 buffer (50 mM, pH = 7). Valve switch every 3 minutes.
587
588 **Figure 4:** hNDPK bioreactor; monitoring of EIC for dTDP and dTTP (m/z 401.0166 $[M-H]^-$ for dTDP
589 and m/z 480.9831 $[M-H]^-$ for dTTP) for (a) denatured and (b) active bioreactors. [dTDP] = 20 µM
590 ([ATP] = 150 µM, $[MgCl_2]$ = 200 µM), flow rate = 40 µL min^{-1}, mobile phase: ammonium acetate
591 buffer (50 mM, pH = 7). Valve switch: 5 minutes.
592
593 **Figure 5:** enzymatic cascade; monitoring of EIC for dTMP, dTDP and dTTP (m/z 321.0500 $[M-H]^-$
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596 min^{-1}, mobile phase: ammonium acetate buffer (50 mM, pH = 7). Valve switch: 5 minutes.
597 **Figure 6:** stability of bioreactors. Conversion efficiency: hTMPK was used 21 times (795 minutes,
598 37°C) over 60 days (diamonds) and hNDPK was used 20 times (915 minutes, 37°C) over 68 days
599 (squares).
600
601 **Figure 7:** Active and denatured hTMPK bioreactor; monitoring of EIC for dTMP (m/z 321.0500),
602 ANP-CH_3-MP (m/z 259.0562) and ANP-Br-MP (m/z 322.0561) conversion into dTMP (m/z
603 401.0166), ANP-CH_3-DP (m/z 339.0150) and ANP-Br-DP (m/z 402.0149). Solution of 20 µM of each
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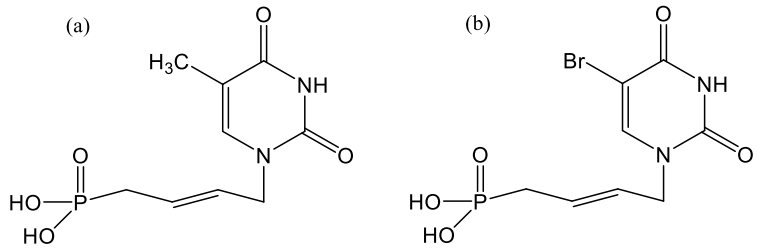
605
606 **Figure 8:** Active *vv*TMPK bioreactor; monitoring of EIC for dTMP (m/z 321.0500), ANP-CH_3-MP
607 (m/z 259.0562) and ANP-Br-MP (m/z 322.0561) conversion into dTMP (m/z 401.0166), ANP-CH_3-
608 DP (m/z 339.0150) and ANP-Br-DP (m/z 402.0149). Solution of 20 µM of each substrate ($[MgCl_2]$ =
609 200 µM, [ATP] = 150 µM, 50 mM $AcNH_4$, pH = 7). Flow rate = 60 µL min^{-1}.
610

Table captions

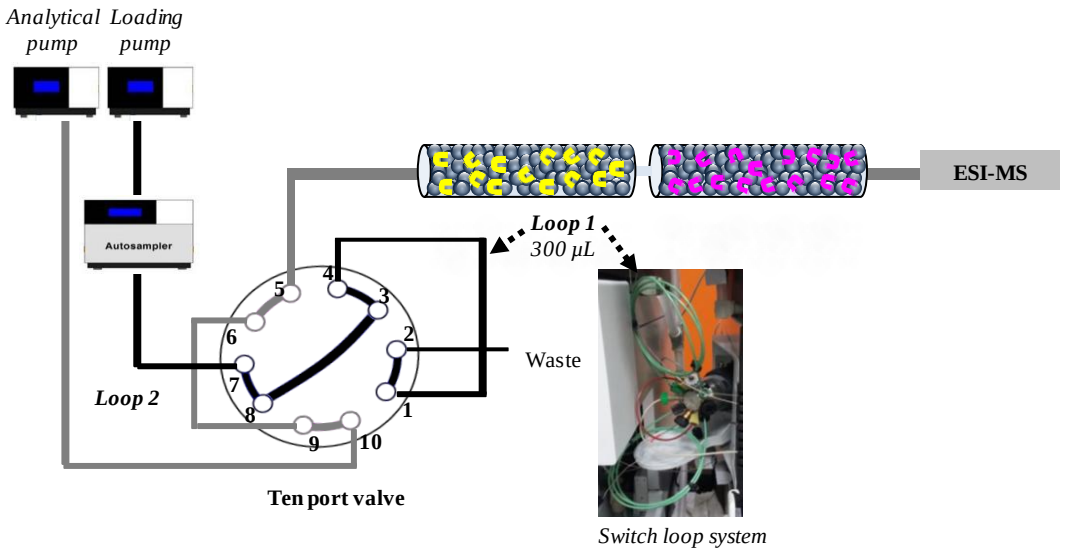
Table 1: m/z values of Thymidine Monophosphate (dTMP), Thymidine Diphosphate (dTDP), Thymidine Triphosphate (dTTP), methyl allylphosphonate uracil (ANP-CH3) and bromine allylphosphonate uracil (ANP-Br).



ANP-CH_3-MP
m/z 259.0562
$[M-H]^-$

ANP-Br-MP
m/z 322.0561
$[M-H]^-$

Figure 1: Chemical formula of (a) Acyclic Nucleoside Phosphonate-CH_3 (ANP-CH_3-MP) and (b) Acyclic Nucleoside Phosphonate-Br (ANP-Br-MP).



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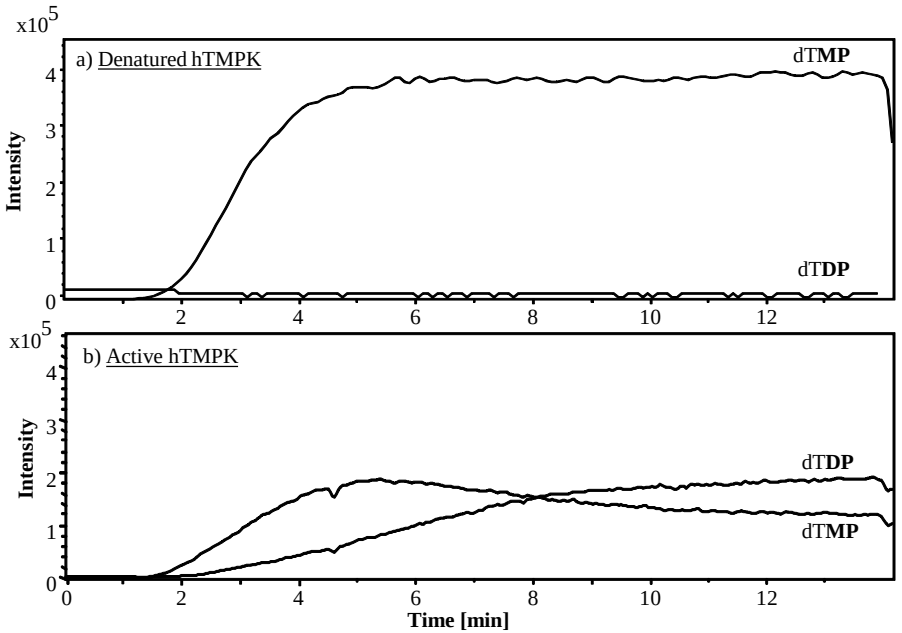

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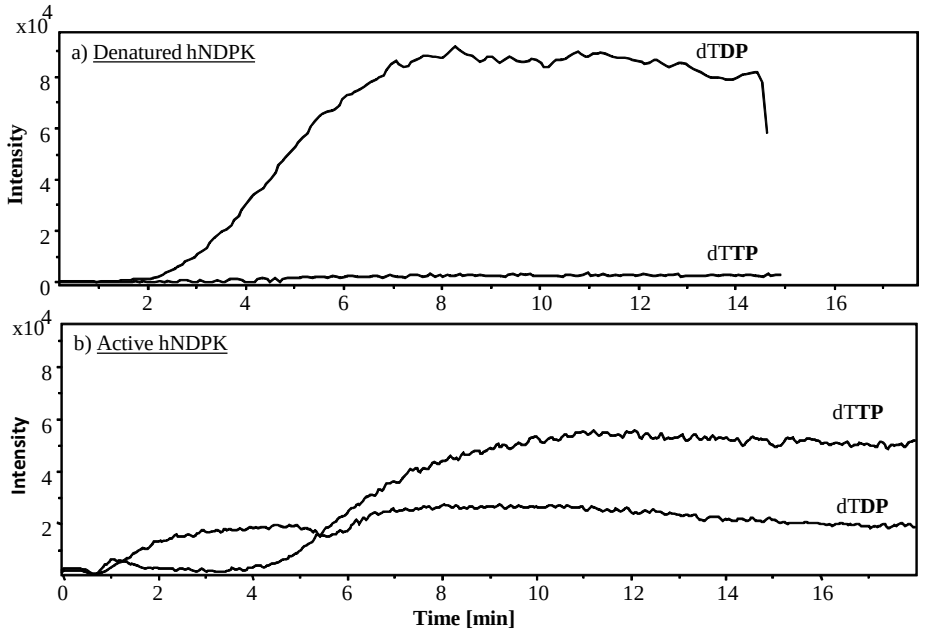

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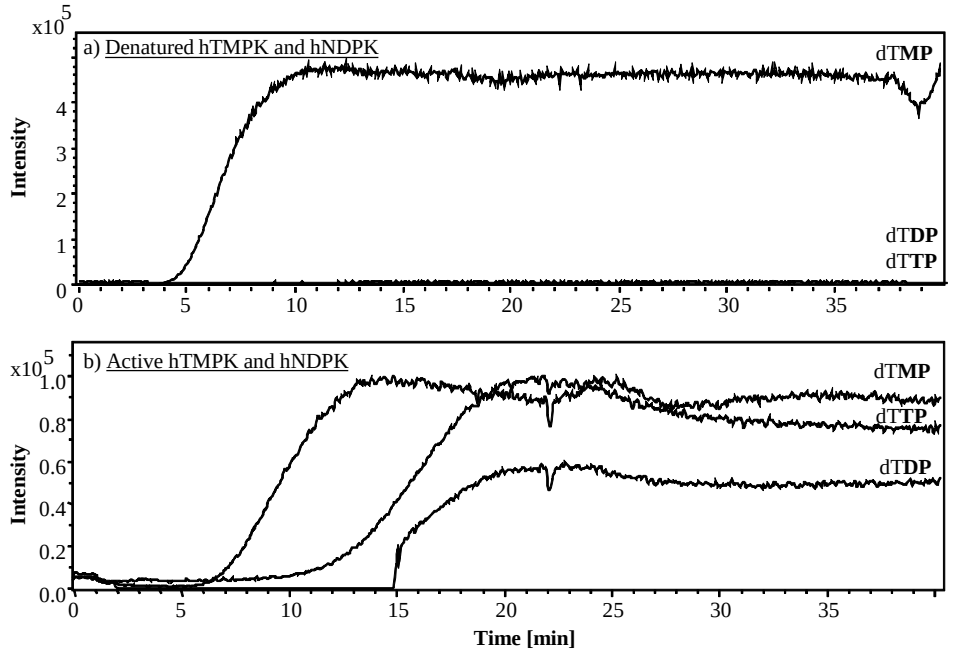

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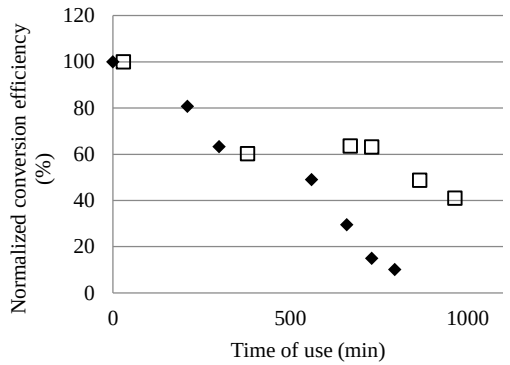

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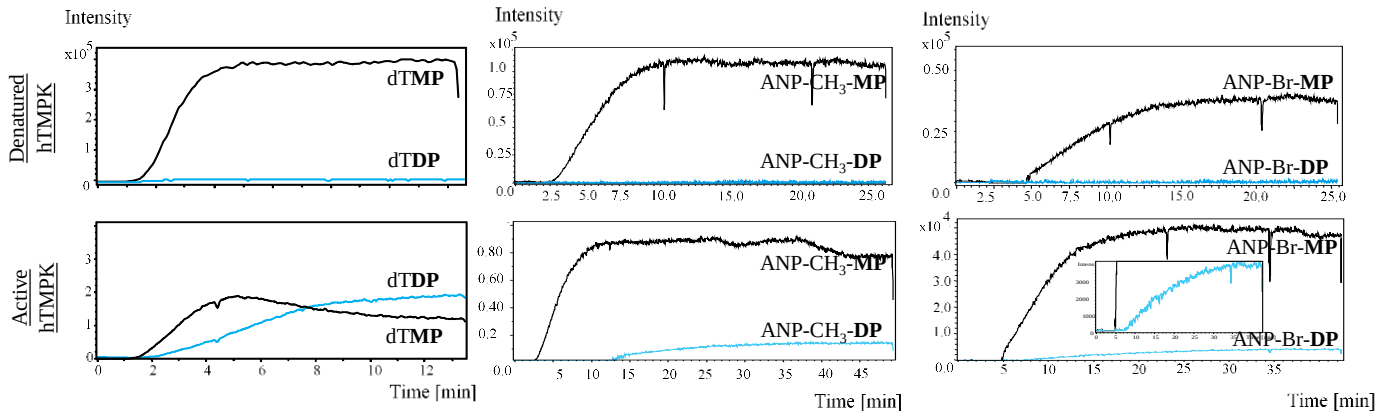

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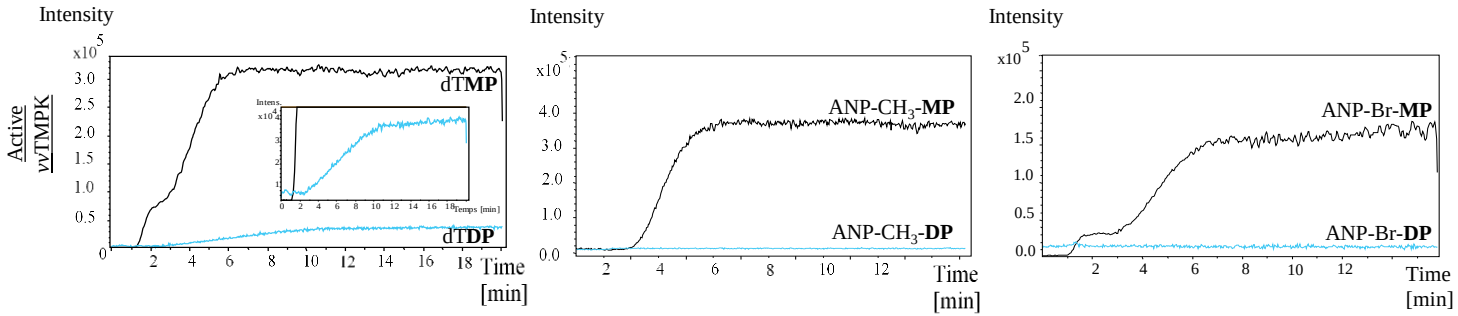
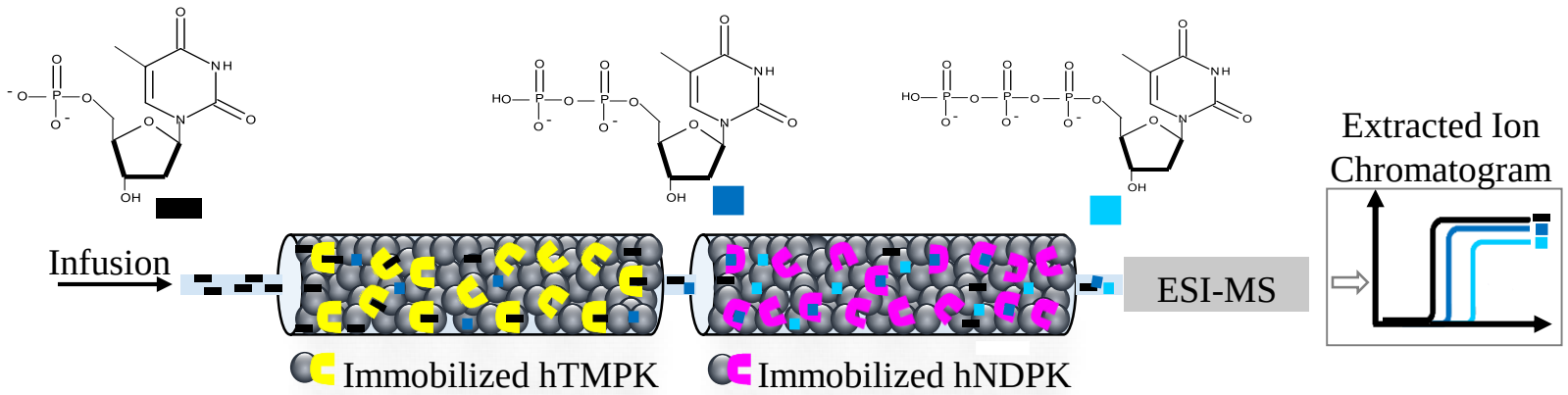


Figure 8: Active *vv*TMPK bioreactor; monitoring of EIC for dTMP (*m/z* 321.0500), ANP-CH_3-MP (*m/z* 259.0562) and ANP-Br-MP (*m/z* 322.0561) conversion into dTMP (*m/z* 401.0166), ANP-CH3-DP (*m/z* 339.0150) and ANP-Br-DP (*m/z* 402.0149). Solution of 20 µM of each substrate ([$MgCl_2$] = 200 µM, [ATP] = 150 µM, 50 mM $AcNH_4$, pH = 7). Flow rate = 60 µL min^{-1}.

		Endogenous		
$[M-H]^-$	dTMP	dTDP	dTTP	
m/z	321.0500	401.0166	480.9831	
		ANPs		
$[M-H]^-$	ANP-CH_3-MP	ANP-CH_3-DP	ANP-Br-MP	ANP-Br-DP
m/z	259.0562	339.0150	322.0561	402.0149

Table 1: *m/z* values of Thymidine Monophosphate (dTMP), Thymidine Diphosphate (dTDP), Thymidine Triphosphate (dTTP), methyl allylphosphonate uracil (ANP-CH3) and bromine allylphosphonate uracil (ANP-Br).


Infusion
Immobilized hTMPK
Immobilized hNDPK
ESI-MS
Extracted Ion Chromatogram