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Optimization of cellular nucleotide extraction and sample preparation for nucleotide pool analyses using capillary electrophoresis

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Abstract

Cell extraction and further sample preparation for nucleotide pool analysis using capillary electrophoresis was faster and simpler using volatile extraction solvents (e.g. organic solvents and de-ionized water) compared to the commonly applied acids dissolved in water (e.g. perchloric acid and trichloracetic acid). Temperature had to be controlled during the whole sample preparation process to prevent degradation, and extracts had to be cleaned from proteins and other large molecules prior to capillary electrophoretic analysis to improve reproducibility. Capillary electrophoresis using borate and cyclodextrins in the background electrolyte was used for determining 11 cellular nucleotides simultaneously. In order to optimize the assay, 0–100% acetonitrile, 0–100% ethanol, and 0–100% methanol in de-ionized water were applied to extract nucleotides from mouse lymphoma cells, and nucleotide yields, recovery, and reproducibility were compared. The assay met the commonly accepted validation limits for biological fluids, if 20–80% acetonitrile in water and 40–60% ethanol in water were used as extraction solvents.

Keywords: Nucleotides

1. Introduction

Nucleotide pool analysis is an important tool in clinical and pharmaceutical research [1]. Treatment of mammalian cells with antiviral and chemotherapeutic drugs can result in perturbations of nucleotide pools [2]. Monitoring these perturbations in both healthy and diseased cellular metabolism is useful in assessing the toxicity of drug therapy and better understanding the mechanism of action of these drugs [1,3]. However, repetitive analysis is required to meet the commonly accepted validation limits for biological fluids [4,5]. Thus, the assay needs to be as simple as possible.

Traditionally, anion-exchange HPLC, reversed-phase HPLC, and ion-pair reversed-phase HPLC were used as the routine method for nucleotide pool analysis [6–8]. Another technique used is capillary electrophoresis (CE) and is known to be a powerful tool for the analysis of cellular nucleotides [9]. One advantage of CE over HPLC is that only small sample volumes are required [10,11]. Additionally,

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CE methods using a pseudo-stationary phase such as cyclodextrins or micelles have been proven to have higher efficiencies than HPLC methods [2,12–14]. Perhaps the main disadvantage of CE compared to HPLC is that CE generally has poorer concentration detection limits when using photometric detection, because of the small inner diameter of the capillary. However, Britz-McKibbin et al. [15,16] improved detection sensitivity of CE methods through an on-line sample concentration technique studying weakly acidic analytes. Large sample volumes were injected while keeping an appropriate pH difference between sample plug and the background electrolyte (BGE). The weakly acidic analytes focused at the pH-junction according to their pK_a, and were studied in the low nano-molar range using a commercial UV detector [16]. Cell extracts analyzed by this method were dried and dissolved in a specific buffer solution prior to injection in order to create the pH junction [15]. Cells were extracted with 50% ethanol (EtOH) that allowed a simple sample preparation, as EtOH evaporated during freeze drying.

Commonly, perchloric acid, trichloracetic acid, formic acid, or tetra butyl-ammonium sulphate dissolved in water were used as extraction solutions for nucleotide extraction from various kind of cells [6,7,17–20]. Major disadvantages of these strongly acidic extraction solvents include cumbersome removal of the extraction solvents from the extracts, and degradation of nucleotide tri-phosphates making it difficult to assay nucleotide tri-phosphate pools [21,22].

The aim of this investigation was to determine the best working volatile extraction solvent for nucleotide pool analysis of mouse lymphoma cells. Therefore, 100% ACN, 100% MeOH, 100% EtOH, and 20, 40, 60, and 80% of these organic solvents in de-ionized water, as well as 100% de-ionized water were systematically compared considering nucleotide yields, recovery, and reproducibility of the assay.

2. Experimental

2.1. Materials

Sodium dodecyl sulfate (SDS), Borax, hydroxypropyl-β-cyclodextrin, pluronic F-68, *N*-[hydroxyethyl]piperazine-*N*′-[2-ethanlsulfonic acid] (HEPES)-buffer, glutamate, heat inactivated horse serum, sodium pyruvate and the following nucleotides: adenosine-5′-mono-, di- and triphosphate (AMP, ADP, ATP), guanosine-5′-mono-, di- and triphosphate (GMP, GDP, GTP), cytosine-5′-mono-, di- and triphosphate (CMP, CDP, CTP), and uridine-5′-mono-, di- and triphosphate (UMP, UDP, UTP) were from Sigma–Aldrich (Oakville, ON, Canada). RPMI 1640 medium lacking glutamine and Trypan Blue dye were from Gibco/BRL Life Technologies (Grand Island, NY, USA). Ethylenediaminetetraacetic acid (EDTA) was from BDH Chemicals (Toronto, ON, Canada). Distilled water was further deionized with a Water-I system from Gelman Science (Ann Arbor, MI, USA). Acetonitrile (ACN), methanol (MeOH), and ethanol (EtOH) were from Merck (Darmstadt, Germany). Phosphate-buffered saline (PBS) stock solution, which contained 80 g/l NaCl, 2 g/l KCl, 11.5 g/l Na_2HPO_4, and 2 g/l KH_2PO_4 was from Fluka (Buchs, Switzerland), and was diluted by a factor of 10 prior to its application. Penciclovir (PCV, 9-(4-hydroxy-3-(hydroxymethyl)but-1-yl)guanine) was kindly provided by SmithKline Beecham (Hertfordshire, UK). Frozen stock of the mouse lymphoma 1210 (ML1210) cell line was obtained from the laboratory of Professor Julia Levy, Department of Microbiology and Immunology of the University of British Columbia.

2.2. Equipment and procedure

2.2.1. Cell cultivation and extraction procedure

Frozen stock of ML1210 cells (2×1 ml) were diluted in 2×20 ml RPMI 1640 medium that contained 0.05% pluronic F-68, 10 m*M* HEPES, 10 m*M* glutamate, 10% heat-inactivated horse serum, and 0.5 m*M* sodium pyruvate. The cells were cultured in a humidified incubator from Forma Scientific (USA), at 37 °C in 5% CO_2 obtained from Praxair (Mississauga, ON, Canada). All cell culture work was carried out in Falcon plasticware (Frankling Lakes, NJ, USA) in a sterile environment using aseptic techniques. The selected ML1210 cell line [23–25] reached exponential growth phase at densities between 0.4 and 1.2 million cells/ml and had an estimated doubling time of 15 h. Following 3 days of incubation, the RPMI 1640 medium was changed in both flasks. This incubation and change of medium

was repeated once. The cultures were then diluted to 2×100 ml with fresh RPMI 1640 medium and incubated for a further 3 days. The 2×100 ml were then diluted to 2×500 ml with fresh RPMI 1640 medium and again incubated for 3 days. The 2×500 ml cells were then pooled and counted using a haemocytometer from Hausser Scientific (USA) and Trypan Blue dye. Finally, 2000 ml suspension culture with a density of 0.8 million cells per ml was obtained by diluting an appropriate volume of the pooled and counted suspension culture to 2000 ml total volume with fresh RPMI 1640 medium and incubating this diluted suspension culture for 15 h.

After 15-h incubation, the extraction procedure was carried out. Each cell extraction used 10 million cells, harvested from the final 2000 ml suspension culture, and a total of 10 extractions were prepared for each type of extraction solvent; nine repetitions were carried out for each solvent type with the 10th sample being reserved for imaging the cells using an Olympus IX-70 microscope at 40-fold magnification. To obtain samples containing 10 million cells, the 2000 ml suspension culture was counted again and volumes corresponding to 10 million cells were transferred into 15-ml tubes. Cells were packed by centrifugation at 960 rpm for 5 min in a Dynac centrifuge from Dickison and Company (Parsippany, NY, USA). Medium was decanted and cell pellets were re-suspended in 2 ml ice-cold PBS solution with gentle vortex shaking. Cells were centrifuged again at 960 rpm for 5 min and PBS solution was decanted. Penciclovir (10 μl of 0.72 *M* stock solution), ATP (20 μl or 40 μl of 0.20 *M* stock solution), and 2 ml extraction solvent were added to the cell pellets with rigorous vortex mixing for 15 min. One sample was used for imaging the cells. Nine samples were centrifuged at 1550 rpm for 5 min, supernatants were transferred into clean 15-ml tubes, and 5 ml de-ionized water was added to each sample to reduce the fraction of organic solvent in the sample solution to <30%, and raise the freezing point of the samples to above −20 °C. Samples were kept at −86 °C until lyophilization in a TiPhilizer™ MP from FTS Systems (Stone Ridge, NY, USA) at −20 °C, for 24 h.

2.2.2. Capillary electrophoretic analysis

The lyophilized samples were dissolved in a 200 μl solution of 0.05 *M* sodium chloride, 0.02 *M* EDTA, pH 7, and ultra-centrifuged through a 5000 Da filter (Millipore, USA), using a Micromax centrifuge from IEC (Mudham Heights, MA, USA) at 13 000 rpm for 60 min. Separations were carried out using an MDQ automated capillary electrophoresis system from Beckman Instruments (Mississauga, ON, Canada). Fused silica capillaries from Polymicro Technologies (Phoenix, AZ, USA) with an I.D. of 75 μm, an O.D. of 368 μm, and a total length of 60.0 cm were used. New capillaries were rinsed with 1 *M* NaOH (10 min, 20 p.s.i.). Each separation was preceded by the following wash sequence: 0.06 *M* SDS (4 min, 20 p.s.i.), de-ionized water, 1 *M* NaOH, de-ionized water (each for 2 min at 20 p.s.i.), and finally BGE (4 min, 20 p.s.i.). The BGE contained 0.16 *M* borate (0.04 *M* Borax), 0.10 *M* hydroxypropyl-β-cyclodextrin, and 0.002 *M* EDTA at pH 9.5. Injection time was 30 s at 0.5 p.s.i., and samples were separated at 30 kV under normal polarity. The sample storage tray was maintained at 4 °C while the temperature of the separation capillary was maintained at 20 °C. Absorbance was monitored using a Beckman PDA-detector at 254 nm. Data were collected using the MDQ P/ACE software from Beckman.

3. Results and discussion

3.1. Extraction and sample preparation

A CE method employing dynamic complexation of nucleotides with hydroxypropyl-β-cyclodextrin and borate was used to analyse the samples [26–28]. A pH difference of 2.5 pH units between sample plug (pH 7.0) and BGE (pH 9.5) was created to focus the weakly acidic analytes at the pH junction as described in previous studies [15,16]. Injection time for the cellular extracts was limited to 30 s at 0.5 p.s.i., as band broadening and poor resolution were observed with longer injection times due to the complex sample matrix.

In cellular organisms, nucleotide tri-phosphates typically have a higher concentration than nucleotide di-phosphates and nucleotide mono-phosphates, as they are the main compounds for nucleic acid synthesis and cellular energy metabolism. ATP should represent the largest signal, as it is the most

important energy carrier in a cell. To maintain the stability of nucleotide tri-phosphates, rigorous temperature control at all times during sample preparation was required (extractions were carried out on ice and the centrifuge was maintained at 4 °C). Temperature control prevented degradation, especially de-phosphorylation of nucleotide tri-phosphates to di-phosphates and mono-phosphates. Ultra-centrifugation of the dissolved samples was necessary prior to CE-analysis in order to stabilize the electroosmotic flow (EOF). Filtering the samples removed proteins and other macromolecules that would interact with the capillary wall and alter its net negative charge. A decreasing net negative charge of the capillary wall caused a decreasing EOF and in turn increasing analyte migration times with poorly resolved peaks. After ultra-centrifugation, changes in the EOF with varying extraction solvent types were much less and most likely because of the reduced amount of large molecules in the sample matrix.

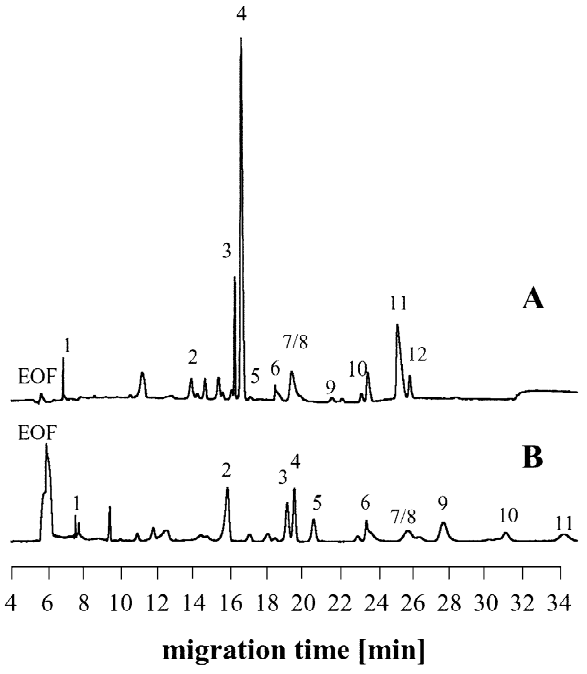

Fig. 1. Separation of nucleotides extracted from 10 million mouse lymphoma (ML1210) cells using 50% EtOH. (A) Sample temperature was maintained below 4 °C throughout sample preparation, and filtered through a 5000 Da filter prior to CE analysis. (B) Temperatures varied from 4 to 15 °C, samples were not filtered. BGE: 160 m*M* borate, 100 m*M* hydroxypropyl-β-cyclodextrin, 2 m*M* EDTA, pH 9.5. Sample buffer: 50 m*M* NaCl, 20 m*M* EDTA, pH 7. Capillary: 75 μm I.D.; 58.5 cm (50 cm eff.); Voltage: 30 kV; Detection: direct at 254 nm, Bandwidth: 10 nm; Injection: 30 s at 0.5 p.s.i. Peak identification: 1, Penciclovir (PCV); 2, AMP; 3, ADP; 4, ATP; 5, GMP; 6, CMP; 7/8, GTP/GDP; 9, UMP; 10, CTP; 11, UTP; 12, UDP.

Fig. 1 shows two electrophoretic analyses of cellular extracts prepared with 50% EtOH as the extraction solvent [15]. Signals were identified by spiking the sample with nucleotide standards and by spectral analysis using a PDA detector. The sample in Fig. 1A was prepared under strict temperature control (<4 °C), and with ultra-centrifugation prior to CE analysis. Without maintaining the extracts below 4 °C during sample preparation, the amount of extracted nucleotide mono-phosphates except CMP increased and exceeded the amount of tri-phosphates (Fig. 1B). If samples were not ultra-centrifuged, migration times of the later emerging signals were much longer. UDP did not pass the detector before 40 min, and UTP was better resolved in the ultra-centrifuged sample.

3.2. Evaluation of best working extraction solvents

3.2.1. Recovery of ATP and penciclovir

ATP was chosen as a reference standard, because it represents the highest signal in the electropherogram when the optimized extraction procedure is used. This resulted in good signal reproducibility (RSD<15%), which is essential for calculating standard additions. ATP served as reference standard to monitor losses of tri-phosphates that occur after extraction from ML1210 cells and before CE-injection. These losses were caused by degradation during sample preparation.

The nucleoside analogue, PCV, was chosen as a reference standard to calculate analyte losses that were not caused by degradation. PCV does not carry phosphate groups and therefore degradation was not expected.

In order to calculate losses of the reference standards, nine samples were prepared for each extraction solvent. Each of the nine samples contained 2 nmol/10 million cells PCV. Beyond this, three samples were spiked with 8 nmol/10 million cells ATP, three samples were spiked with 4 nmol/10 million cells ATP, and three samples were not spiked with ATP. The reference standards were

added to the cells together with the extraction solvent. The amount of cellular ATP was determined from the last three samples. The average amount of cellular ATP was subtracted from the amount of ATP determined in the samples spiked with 8 and 4 nmol/10 million cells ATP, respectively. Recoveries were calculated by external standard calibration based on corrected peak areas (peak area divided by migration time). Corrected peak areas were used for the calculation as they do not depend on changes in the EOF.

Table 1 lists recoveries and RSD values for PCV and ATP calculated for the different extraction solvents. Extracting with 100% ACN, PCV was recovered with 109±14%, but no ATP was detected in the samples. Poor solubility of ATP in ACN was most likely responsible for the lack of ATP in the spiked samples.

Accuracy was poor for 100% de-ionized water, 20–100% MeOH in water, and 80% EtOH in water. Using 100% EtOH, 20–60% EtOH in water, and 20–80% ACN in water, accuracy was within the commonly accepted limits of ±15% for biological fluids [5] and these solvents were favoured as extraction solvents.

3.2.2. Extraction solvent and nucleotide yields

A comparison of 100% ACN, 100% MeOH, 100% EtOH, and 20, 40, 60, and 80% of these organic solvents in de-ionized water, as well as 100% de-ionized water as extraction solvents for nucleotide pool analysis was done with a focus on nucleotide tri-phosphates. The values for GTP represent the amount of extracted GTP and GDP in total, as these two signals overlapped (Fig. 1). Nucleotide yields (nmol/10 million cells) were based on corrected peak areas, calculated in μ*M* by external standard calibration, and multiplied by 2×10^{-3} l, in order to obtain values that correspond to 10 million cells; 2×10^{-3} l was the sample buffer volume in which the freeze dried samples were dissolved prior to CE analysis.

Table 2 lists concentrations and RSD values for ATP, GTP/GDP, CTP, and UTP determined from the nine samples/extraction solvent prepared for calculating recoveries of ATP and PCV in Section

Table 1
Recoveries (%) and RSD values (%) of the reference standards penciclovir (PCV) and ATP

	PCV (% recovery)	RSD (%, $n=9$)	ATP (% recovery)	RSD (%, $n=6$)
100% ACN	109	14	Not detected	
100% EtOH	108	10	97	14
100% MeOH	79	12	82	14
100% de-ionized water	112	14	81	12
20% EtOH	100	15	87	24
40% EtOH	114	14	88	11
60% EtOH	108	7	92	14
80% EtOH	104	8	80	11
20% MeOH	84	10	87	14
40% MeOH	81	10	81	14
60% MeOH	82	15	81	11
80% MeOH	78	15	83	14
20% ACN	108	13	96	9
40% ACN	106	14	105	18
60% ACN	116	15	89	10
80% ACN	111	13	88	13

Extractions were carried out with acetonitrile (ACN), ethanol (EtOH), methanol (MeOH), and organic solvent/de-ionized water mixtures.

Table 2
Nucleotide yields (nmol/10 million cells) and RSD values (%) obtained with acetonitrile (ACN), ethanol (EtOH), methanol (MeOH), and organic solvent/de-ionized water mixtures for nucleotide tri-phosphates (ATP, GTP/GDP, CTP, UTP)

	ATP (nmol/10 million cells)	RSD (%, n=3)	GTP/GDP (nmol/10 million cells)	RSD (%, n=9)	CTP (nmol/10 million cells)	RSD (%, n=9)	UTP (nmol/10 million cells)	RSD (%, n=9)
100% ACN	Not detected		Not detected		Not detected		Not detected	
100% EtOH	Not detected		Not detected		Not detected		Not detected	
100% MeOH	10.3	14	0.3	37	0.2	64	2.8	23
100% De-ionized water	12.2	12	1.0	16	0.3	48	3.3	22
20% EtOH	9.6	14	0.7	24	0.3	24	2.4	28
40% EtOH	12.9	7	1.1	10	0.3	21	4.5	14
60% EtOH	13.5	7	1.1	17	0.4	29	4.7	8
80% EtOH	8.2	6	0.4	24	0.3	22	2.3	12
20% MeOH	17.5	14	1.4	36	0.3	44	4.7	42
40% MeOH	15.9	8	1.7	13	0.4	44	5.3	15
60% MeOH	16.6	5	1.7	10	0.4	19	5.7	9
80% MeOH	17.8	12	1.5	15	0.2	48	6.0	13
20% ACN	14.8	5	1.2	18	0.4	36	5.4	13
40% ACN	16.9	7	1.4	11	0.3	45	6.2	10
60% ACN	15.0	6	1.4	8	0.3	46	5.6	15
80% ACN	13.5	2	1.1	25	0.2	44	4.9	24

3.2.1. As six of the nine samples were spiked with ATP, only three instead of nine samples remained to calculate RSD values for ATP; 100% ACN and 100% EtOH were not applicable for nucleotide extraction, as no nucleotides were detected. With 100% MeOH, 100% de-ionized water, 20 and 80% EtOH, low concentrations of cellular nucleotides were detected. Applying 40–60% EtOH in water, 20–80% MeOH in water, and 20–80% ACN in water resulted in higher nucleotide yields.

In order to determine the reasons for varying nucleotide yields using the different extraction solvents, the effects of EtOH, MeOH, de-ionized water, and ACN on ML1210 cells after an exposure time of 15 min were investigated visually with an Olympus IX-70 microscope at 40-fold magnification. Results are shown in Fig. 2. Cells treated with alcohol (EtOH, MeOH) (Fig. 2A) coagulated in clusters, but remained the same size as cells suspended in PBS buffer (Fig. 2B). Cells treated with de-ionized water

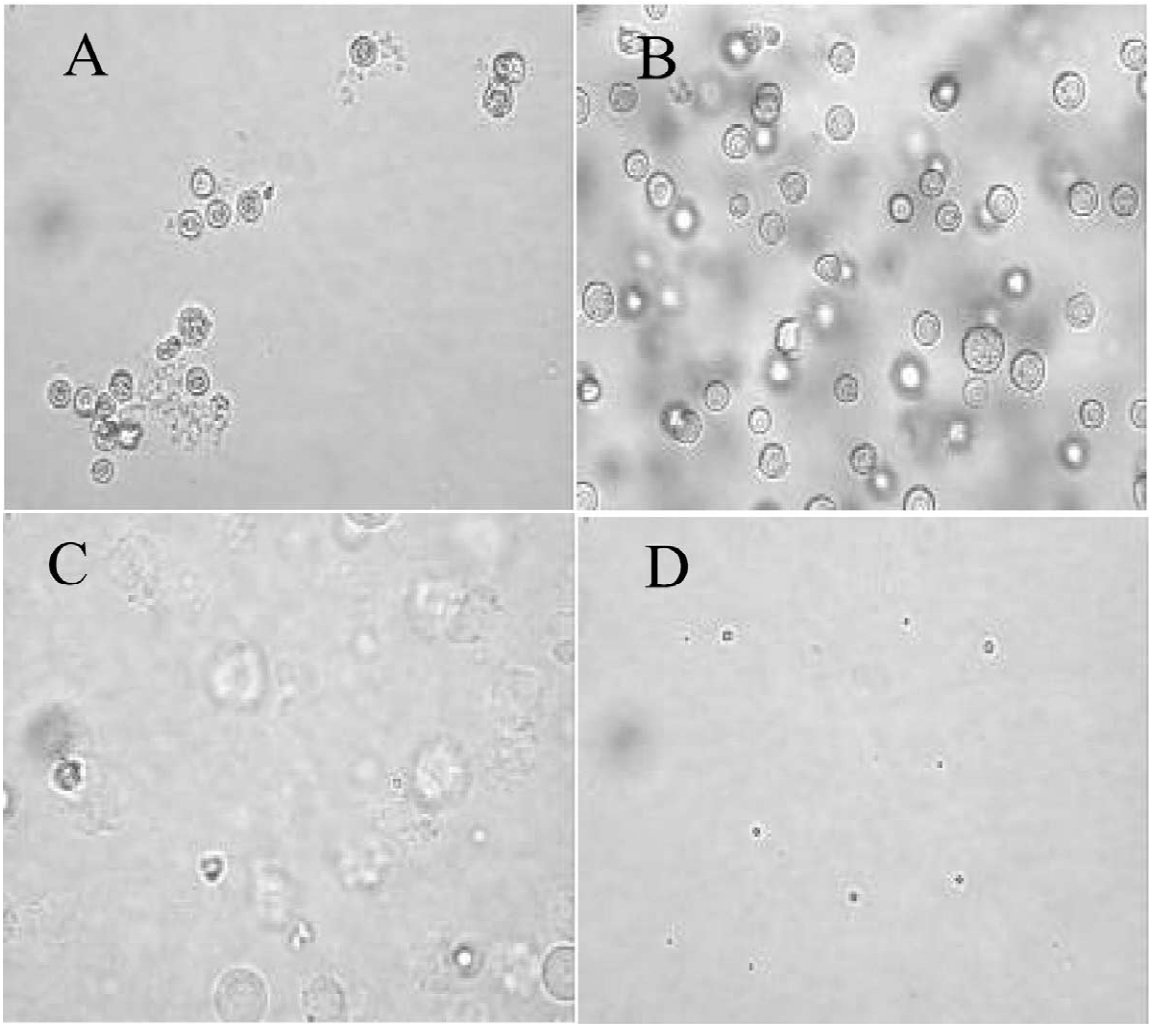

Fig. 2. Pictures of mouse lymphoma (ML1210) cells after 15-min exposure to (A) 100% EtOH; (B) PBS buffer; (C) 100% de-ionized water; (D) 80% ACN. Pictures were taken from a spare sample prepared for each extraction solvent with an Olympus IX-70 microscope at 40-fold magnification.

(Fig. 2C) increased in diameter, whereas cells treated with 80% ACN shrunk (Fig. 2D) until they were hardly visible with 100% ACN.

The different shapes of the cells are most likely due to osmosis and counter-osmosis resulting from exposure to hypotonic solvents. De-ionized water diffuses into cells as a result of osmotic pressure and can cause hypotonic shock. Organic solvents most likely solvate the lipid tails of the phospholipids that compose the cell membrane double layer and holes appear in the cell membrane allowing smaller molecules like nucleotides to pass through.

ACN (100%) is known as an effective protein denaturant [21], and cell membranes might be significantly damaged upon exposure to this solvent, allowing cytoplasm to escape the cells. EtOH and MeOH were less damaging to the cell membranes than ACN, as cells were still in good shape after 15-min exposure to these alcohols. Membrane disruption seemed to be much slower as 15 min was too short a time to extract significant amounts of nucleotides using 100% EtOH and 100% MeOH. Low nucleotide yields were obtained using 100% de-ionized water, as cells did not lyse after 15-min exposure (Fig. 2 C). Using 20 and 80% EtOH as extraction solvent resulted in low nucleotide yields. However, the combination of osmotic shock and membrane disruption seemed to optimize nucleotide extraction, as 40–60% EtOH in water as well as 20–80% MeOH in water and 20–80% ACN in water created the best conditions for the release of cytoplasmic contents.

3.2.3. Reproducibility

For analytes in biological fluids, reproducibility should always fall within the range of ±15%, except at the lower limit of quantitation (LOQ), where it should not deviate by more than ±20% [5]. The LOQ was calculated from the limit of detection (LOD). The LOD for this method was found to be 0.18 nmol/10 million cells (ATP and GTP) and 0.36 nmol/10 million cells (CTP and UTP). Consequently, the LOQ was 0.6 nmol/10 million cells (ATP and GTP) and 1.2 nmol/10 million cells (CTP and UTP). Differences in LOD amongst ATP, GTP, CTP, and UTP existed, because CTP and UTP have smaller molar absorptivities at 254 nm than ATP and GTP. The lower velocity of CTP and UTP also leads to higher LOD, as these signals were less resolved compared to ATP and GTP/GDP, which migrated earlier from the capillary.

RSD values for CTP were exceptionally high for most extraction solvents (Table 2), as CTP was extracted significantly below the LOQ. Changes in CTP concentrations were masked by the poor reproducibility of these signals. Therefore, CTP was not considered in comparing the precision of the different extraction solvents.

Reproducibility was poor for at least one of the remaining three nucleotide triphosphates in addition to CTP (Table 2) when using 100% MeOH, 100% de-ionized water, 20%–80% EtOH in water, 20% MeOH, and 80% ACN. For the remaining solvents (40%–60% EtOH in water, 40%–80%, MeOH in water, and 20%–60% ACN in water), the reproducibility for ATP and UTP was better than 15% and for GTP/GDP better than 20%.

3.3. Recovery of GTP, CTP and UTP

After comparison of recovery, nucleotide yield, and reproducibility, 40–60% EtOH in water and 20%–60% ACN in water were favoured for nucleotide pool analysis of ML1210 cells. Further method validation was carried out for 40% EtOH and included recovery of GTP, CTP and UTP. Losses of GTP, CTP and UTP were determined according to the procedure carried out for ATP (three samples spiked with 40 μ*M* nucleotide, three samples spiked with 20 μ*M* nucleotide, and three unspiked samples). Recoveries were found to be 74±11% for GTP/GDP, 65±15% for CTP, and 69±14% for UTP ($n=6$).

4. Conclusions

The assay for nucleotide pool analysis of cellular extracts was optimized and can be applied to drug treatment experiments in the future. The sample preparation time for nucleotide pool analysis can be decreased through the use of volatile extraction solvents. The degradation of nucleotide triphosphates was prevented by performing cell extractions below 4 °C. The reproducibility was improved by removing proteins and other macromolecules with 5000-Da

cut-off filters from the cell extracts prior to CE analysis. It was observed that a combination of osmotic shock and cell membrane disruption done by organic solvent/de-ionized water mixtures created the best conditions for the release of cytoplasmic contents. Recovery of PCV and ATP and reproducibility data reached the commonly excepted validation limits for biological fluids using 40–60% EtOH in water and 20–60% ACN in water. For future work, a more sensitive detection method is needed to include perturbations of nucleotide di-phosphate pools and nucleotide mono-phosphate pools in the discussion.

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