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Quantitative profiling of nucleotides and related phosphate-containing metabolites in cultured mammalian cells by liquid chromatography tandem electrospray mass spectrometry

Rebecca L. Cordell[a,b], Stephen J. Hill[b], Catharine A. Ortori[a], David A. Barrett[a,*]

[a] Centre for Analytical Bioscience, School of Pharmacy, University of Nottingham, Nottingham NG7 2RD, UK
[b] Institute of Cell Signalling, School of Biomedical Sciences, University of Nottingham, Nottingham NG7 2UH, UK

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ABSTRACT

A method has been developed for the quantitative profiling of over twenty nucleotides and related phosphorylated species using ion-pair reversed-phase liquid chromatography hyphenated to negative ion tandem electrospray mass spectrometry. The influence of mobile phase pH and ion-pairing agent concentration were assessed to optimise separation and peak shapes. Full quantitative analysis was obtained for the nucleotides by reference to structurally related calibration standards. The developed method was applied to profile changes in nucleotides and related compounds in monolayer cultured Chinese hamster ovary (CHO) cells expressing the β_2 adrenoceptor when exposed to pharmacological stimuli. These experiments demonstrate the potential of the LC–MS/MS method to detect changes in nucleotide drug targets as well as the simultaneous monitoring of levels of other nucleotides.

1. Introduction

Nucleotides and related phosphorylated metabolites have many important roles in biological systems apart from being the major components of nucleic acids. They have roles as intermediates in biosynthetic pathways, e.g. UDP-glucose as a precursor to glycogen, as energy carriers in the case of ATP and GTP, as major adenine-containing co-enzymes: NAD, FAD and CoA and as metabolic regulators controlling enzymes, for example, cAMP, a common mediator for many hormones present in multiple signalling pathways [1]. The intracellular concentrations of nucleotides can be profoundly affected as a result of many drug treatments or in the course of normal cellular function, rapidly changing from sub nM to mM concentrations. A method capable of the simultaneous and quantitative determination of such metabolites at nM concentrations would be a valuable asset in understanding both biochemical and pharmacological mechanisms of drug action. Although many methods have been developed to measure nucleotide levels, few have the capability of comprehensive nucleotide profiling at nM concentrations in small volume or low mg weight biological samples. The ability to measure simultaneous changes in many nucleotides and related compounds is important as it allows the monitoring of unpredicted changes in unrelated pathways as well as within target molecules in target pathways.

Profiling nucleotides and other phosphorylated metabolites in biological systems was analytically challenging because of their chemical similarities, chemical and biological instability, high polarity, anionic nature and, in certain situations, highly variable concentrations. Due to their polar nature nucleotides are weakly retained on a reversed-phase HPLC column. To overcome this problem nucleotides have been traditionally separated by ion-exchange chromatography with some of the first methods reported using this technique in the 1950s [2]. Ion-pair chromatography is an alternative that has been used for a number of years and has seen widespread use [3]. The use of non-volatile ion-pairing agents is still employed successfully, for example, by Huang et al. [4] who separated a mixture of nine nucleoside triphosphates and their deoxy counterparts extracted from tumour cell lines using the ion-pairing agent tetrabutylammonium hydroxide with a water/methanol mobile phase. Giannattasio et al. [5] also used a similar non-volatile ion-pairing agent to achieve good separation. Although HPLC methods coupled to UV detection HPLC [4–9] can be used as stand-alone techniques for nucleotide analysis, they do not have sufficient selectivity and sensitivity for application to biological samples where determination of multiple nucleotides in nmolar concentrations is required. Owing to this LC-ESI-MS has evolved as one of the main bioanalytical methods of choice for such complex bioanalytical applications [10,11] including in the field of nucleotide analysis [12–26]. The high salt content of the ion exchange chro-

* Corresponding author. Tel.: +44 115 951 5062; fax: +44 115 951 5102.
E-mail address: david.barrett@nottingham.ac.uk (D.A. Barrett).

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matography mobile phase is incompatible with LC–MS, as is the use of non-volatile ion-pairing agents so alternative methods have also been developed as alternatives for nucleotide analysis. For example, HPLC with electrochemical detection [27], capillary electrophoresis [28–30] and a number of hyphenated techniques such as CE-MS [18–20]. However, none of these methods to date were used to profile a large range of biochemically relevant nucleotides and related phosphorylated metabolites in cultured mammalian cells.

When combined with ion-pair chromatography, however, LC–MS, can offer a highly sensitive and selective method of measuring nucleotide levels in biological samples. The nature of the ion-pairing agent used is, however, crucial to the success of ion-pair LC–MS. The use of traditional anion pairing agents such as tetralkylammonium salts with MS produces good separation but even in low concentrations such agents cause problems at the MS source because of their lack of volatility [15]. In recent literature volatile ion-pairing agents have been used to improve the compatibility of ion-pair chromatography with electrospray MS [13,14,17,18,24].

Preliminary studies have shown that dimethylhexylamine (DMHA) is one such volatile ion-pairing agent that can be used to provide separation with minimal interference in LC–MS analysis of mixtures of nucleotides [14]. The approach has been shown to be successful in measuring a variety of nucleotides in biological samples including tri-phosphate nucleotides in C6 glioma cells [13] and adenosine-containing nucleotides in the same cells [18], as well as dNTPs in human peripheral blood mononuclear cells [17]. However, the scope of analysis of these methods is limited to a small number of analytes from biological samples, or a larger number but focussing solely on standard solutions.

In this paper we present a further development of the use of DMHA in an ion-pair HPLC tandem electrospray ionisation-mass spectrometry assay method to profile quantitatively more than twenty nucleotides simultaneously, exceeding the scope of present methods of this kind. This method was applied to profile intracellular nucleotides in cultured Chinese hamster ovary (CHO) cells and to study their response to drug treatments.

2. Experimental

2.1. Chemicals

All standards (adenine, adenosine, cAMP, AMP, ADP, ATP, cGMP, GMP, GDP, GTP, UMP, UDP, UTP, CMP, CDP, CTP, NAD, NADH, NADP, NADPH, nicotinic acid-adenine dinucleotide phosphate (NAADP), FAD, FMN, cyclic ADP-ribose (cADPR)), and internal standards (8-bromoadenosine 3′,5′-cyclic monophosphate, 8-bromoadenosine 5′-triphosphate, and nicotinamide 1,N^6-ethenoadenine dinucleotide) as well as isoprenaline and 3-isobutyl-1-methylxanthine (IBMX) were purchased from Sigma–Aldrich (Poole, UK) purities >98%. *N*,*N* dimethylhexylamine (DMHA, 99%), potassium hydroxide (85%) and acetic acid (>99.7%) were purchased from Acros Organics (Geel, Belgium). Methanol (HPLC grade), acetonitrile (HPLC grade), ethanol (96%) and formic acid (99%) were purchased from Fisher (Loughborough, UK). Wheatgerm agglutinin (Alexa Fluor) was obtained from Molecular Probes. In all experiments Maxima water was used (18.2 Ω) and all mobile phases were filtered through a 0.47 μm nylon filter (Watman, Maidstone, UK) before use. For initial MS tuning a 10 μM mixture of all standards and internal standards was made in methanol/water (50:50, v/v). For the standard working solutions for LC–MS measurements 10 μM mixtures of the nucleotides were used dissolved in water, stored in aliquots at −80 °C.

2.2. Instrumentation

An Agilent 1100 HPLC system with autosampler (Agilent Technologies, Waldbronn, Germany) was used. A reversed-phase column 150 mm × 2 mm, 3 μm particle size (Waters Symmetry) maintained at 40 °C was used at a flow rate of 0.2 ml/min. UV detection was carried out at 250 nm. The mobile phase A was water:methanol (95:5), pH 7, and B was water:methanol (20:80) with the addition of DMHA to both mobile phases at a concentration of 0.5–20 mM. Mobile phase pH was adjusted using acetic acid. A gradient elution profile was used from 0 to 53% B at 22 min and then to 100% B at 25 min (held until 35 min).

Mass spectrometry work was carried out on a Micromass Quattro *Ultima* triple quadrupole mass spectrometer (Micromass, Manchester, UK). The source temperature was set at 125 °C and the drying gas (nitrogen) and nebulising gas (nitrogen) were set at 850 and 150 l/h, respectively, in negative ion electrospray mode. Argon was used as the collision gas and the collision energy was optimised for each individual compound. Cone voltages were specific to each compound set to provide a good response for the $[M-H]^-$ ion. Multi reaction monitoring (MRM) measurements were carried out for the detection of the nucleotides in standard solutions and biological samples, with a dwell time of 0.2 s.

2.3. Sample preparation

All biological samples were extracted from cultured Chinese hamster ovary cells (CHO) expressing the β_2 adrenoceptor. CHO cells were cultured in HAM/F12 medium supplemented with 10% (v/v) foetal calf serum and 2 mM glutamine at 37 °C in humidified atmosphere of air:CO_2 (95:5). Cells were grown to confluence in six-well plates, then medium was removed and replaced with 1 ml of extraction solution at −20 °C, containing 10 μM of internal standards. Extraction solvents evaluated were acetonitrile, ethanol, methanol acetonitrile:water (8:2), ethanol:water (8:2), methanol:water (8:2), 0.1 M formic acid, 0.5 M perchloric acid and 0.1 M formic acid in methanol. The extraction solvent was then removed and centrifuged (15,000 × *g* for 5 min) and the supernatant removed. The effect of sonication on ice for 5 min of cells in situ in six-well plates prior to removal of extraction fluid was investigated. In the case of methanol extraction, the effect of adding an equal volume of hexane was investigated, the methanol fraction being removed following shaking. Perchloric acid was neutralised with KOH and the precipitate removed by centrifugation. All samples were then evaporated under nitrogen (40 °C) to dryness and re-dissolved in 50 μl water prior to analysis.

For the drug stimulation studies cells were grown to confluence in six-well plates, then medium was removed and replaced with 1 ml serum free medium (SFM) or isobutylmethylxanthine (IBMX, 10^{-3} M) in 1 ml SFM. The cells were then incubated for 20 min. After this time to half of the IBMX treated plates and half the control plates isoprenaline (100 μl, 10^{-5} M) in SFM was added. To the remaining plates SFM (100 μl) was added and the plates were incubated for a further 10 min. This produced four groups; controls, IBMX stimulated, isoprenaline stimulated and IBMX + isoprenaline stimulated. After this time media was removed and replaced with the pre-cooled (−20 °C) extraction methanol (1 ml containing 10 μM internal standards). The methanol was then centrifuged (15,000 × *g* for 5 min) to remove protein precipitate the analytes were extracted with hexane as described above, evaporated and re-dissolved for analysis.

2.4. Method validation

Method validation was carried out using the methanol/hexane extraction, again with internal standards included in the methanol at 10 μM. Replicate blank matrices ($n = 6$) were analysed, involving the same extraction procedure being carried out on six-well plates incubated with medium but with the absence of cells for endogenous substances which could potentially interfere with the determination of the analytes. Intra-day accuracy and precision were evaluated by analysis of replicate experiments, involving extraction of standards at concentrations of 1 (low), 10 (medium) and 50 μM (high) ($n = 6$ at each level) on the same day. To assess the inter-day accuracy and the precision, replicate spiked samples ($n = 6$) were analysed on 6 different days. The precision was calculated from the relative standard deviation (R.S.D.%) of the replicate analyses. Accuracy was calculated by comparison of expected concentrations with the measured concentration of the spiked samples. A R.S.D.% of 15% was deemed acceptable for both precision and accuracy at the mid and high concentrations and 20% and the lowest concentration. The analyte recovery was calculated by comparing the peak area of the extracted samples to the peak area from the unextracted standard solutions of equivalent concentration prepared in mobile phase. Ion suppression effects were estimated for each analyte at three different concentrations (1, 10 and 50 μM) by the method of standard addition, corrections being made for the endogenous concentration of the analytes.

Calibration standards for each analyte were constructed over the range of 0.5–100 μM in extract ($n = 6$ at each level) using linear regression analysis (no weighting) from plots of the analyte/internal standard area ratio against concentration. Limits of detection were found by injection of 10 μl standard mixture at decreasing concentrations, until signal to noise ratio (S/N) fell to below 3:1 for an individual analyte.

2.5. Calculation of cell volume

In order to be able to express the levels of the nucleotides as actual intracellular concentrations a simple confocal microscopy experiment was carried out to determine the average volume of a cell. The cells were grown for 48 h on Mattek dishes with a coverslip base, using standard culture conditions as described previously. They were incubated with wheatgerm agglutinin, 633 nm (1 μg/ml) for 30 min at 4 °C in HEPES buffered saline, washed briefly in the saline and then imaged immediately at room temperature. The instrument used was a Zeiss LSM510uv META combi confocal on a Zeiss Axiovert 100 microscope. The objective lens used was a Zeiss Plan Neofluar 40×/1.3 oil. Cells were excited with a HeNe 633 nm laser with a LP650 filter and z stacks taken at 0.5 μM intervals and individual cell volumes calculated from the z stack data. The number of cells per well of the six-well plate was determined by growing cells in six-well plates as described above, with nine wells (from 3 different plates) chosen at random, cells stained with Trypan Blue and viable cells counted using a haemocytometer.

3. Result and discussions

3.1. HPLC development

The effect of differing concentrations of the volatile ion-pairing agent DMHA on the peak shapes and resolution of a standard test mixture of adenine-containing compounds was investigated (Fig. 1). A concentration of DMHA of below 0.5 mM resulted in poor separation and broadened peak shapes confirming similar observations reported by Tuytten et al. [14]. At a concentration of 0.5 mM DMHA little improvement in peak shape or column retention was observed, however, retention times of all the analytes increased as the ion-pairing agent was increased from 0.5 through to 5 mM. At DMHA concentrations of greater than 5 mM only minor changes in retention behaviour of the nucleotides occurred. The use of DMHA has been reported to have adverse effects on sensitivity [13] when using mass spectrometry detection so 5 mM, the lowest concentration still giving good separation, was chosen as the optimal concentration. DMHA has also been used as an ion-pairing agent in related investigations by Tuytten et al. [14] who also found that 5 mM DMHA gave the best balance of chromatographic separation and mass spectrometry sensitivity. Cai et al. [13] recommended that concentrations of DMHA higher than 5 mM were necessary for good separation, but this was not found to be the case in our investigations. In other studies, higher DMHA concentrations have been used successfully, for example, Qian et al. used 8 mM [18], Auriola et al. 10 mM [19] and Fung et al. reported that a concentration of 20 mM [20] was necessary for efficient separation.

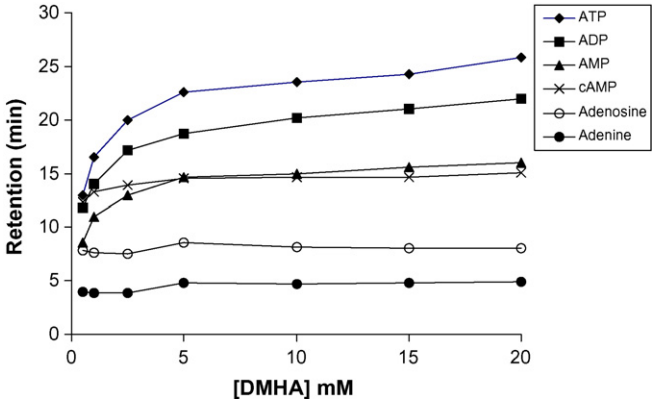

Fig. 1. Effect of increasing DMHA concentration (mM) in mobile phase on retention times of adenine group of nucleotides. Chromatographic conditions: mobile phase A, 0.5–20 mM DMHA in water/methanol (95:5) adjusted with acetic acid to pH 7; mobile phase B, 0.5–20 mM DMHA in water/methanol (20:80). Gradient elution 0–22 min 0–55% B, 22–25 min 55–0% B, 25–35 min 0% B. Column temperature 40 °C.

The effects of mobile phase pH on retention times and peak shapes of a selected group of the nucleotides were also examined. Retention times generally increased as mobile phase pH increased which can be explained by changes in the ionisation of the phosphate moiety increasing ion-pair formation. At lower mobile phase pH values there was also degradation in peak shape, with peaks broadening as the pH decreased. At pH 3 the compounds were barely retained on the column and all eluted at very similar times. In addition, at pH 3 the nucleotide triphosphates and diphosphates were unstable and readily lost one or two phosphate groups, an effect also noted by Cai et al. [13]. From these experiments a mobile phase of pH 7 was found to be most favourable in terms of stability, resolution and peak shapes and is also the pH used in several other nucleotide separation methods involving the use of DMHA [13,18,20]. The final HPLC method provided a balance of MS compatibility with a baseline separation of the majority of the 24 components within a 25 min analysis time (Fig. 2).

3.2. Mass spectrometry development

The potential of both ESI^+ and ESI^- MS were evaluated for detection of the nucleotides. Although ESI^- would seem to be the logical choice for the analysis of negatively charged compounds such as nucleotides there is evidence to suggest that, with the addition of the ion-pairing agent DMHA, it is possible to use ESI^+ to detect

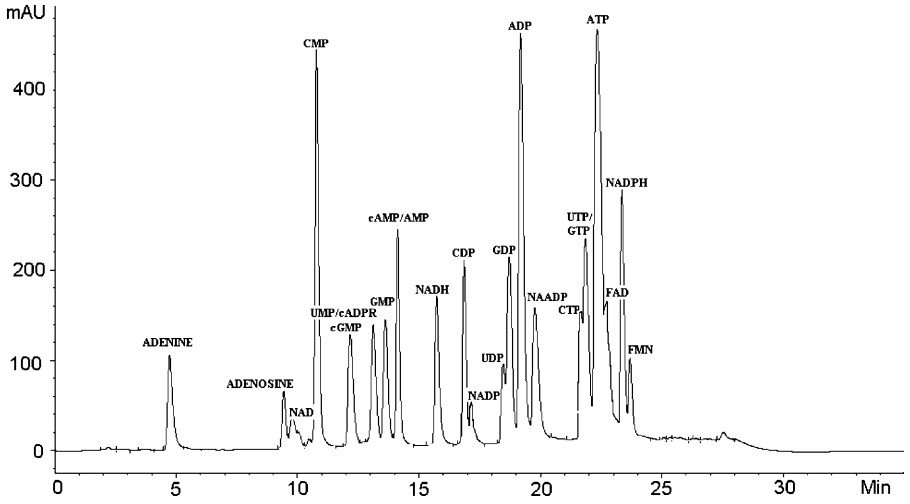

Fig. 2. Optimised HPLC separation of 24 nucleotides in standard solution 10 μM.

the adduct ions that form between the ion-pairing agent and the nucleotides. This effect was observed by Tuytten et al. [14] who described clearer spectra with increased intensities of the adduct ion in ESI^+ rather than the $[M-H]^-$ ion in ESI^- when using DHMA in the mobile phase. Fung et al. [20] analysed the carbocyclic nucleoside analogue Ziagen and its phosphorylated metabolites also using ESI^+ LC–MS with DMHA. Here they found that in ESI^- mode sensitivity was insufficient to detect the triphosphate metabolite of Ziagen, and Ziagen had a poor MS response in this mode. Soga et al. [31], however, developed a CE-ESI-MS method using ESI^- which is capable of monitoring various nucleotides and anionic metabolites.

In order to determine the optimum operation mode for this application all compounds were examined individually in ESI^+ and ESI^- modes (see ADP as an example in Fig. 3) by direct injection into the mass spectrometer of a 10 μM standard mixture. In ESI^- mode the most abundant ion seen was the $[M-H]^-$, with low levels of sodium and potassium adducts present also, whereas in ESI^+ mode the $[M+H]^+$ was not most abundant. In ESI^+ the spectra produced were more complex with high background interference from the protonated DMHA ion and the production of many other fragment ions. The most abundant relevant ion was the adduct ion between the compound and the ion-pairing agent $[M+DMHA]^+$. Due to the increased distribution of the total ion content sensitivity in ESI^+ mode was notably reduced compared with that seen in ESI^- and in ESI^- the intensities of the $[M-H]^-$ ions generally were stronger than those observed for the $[M+DMHA]^+$ ion in ESI^+. In ESI^- mode there was a low background and all the analytes could be detected, whereas with ESI^+, adenine, adenosine, cGMP, GMP, UDP, UTP and CTP failed to produce a signal. The main difficulty with using ESI^+ mode with DMHA was that DMHA itself produced a very strong signal that suppressed other positive ions. Due to the simple and uncluttered spectra produced, ESI^- mode was chosen to carry out further MS work, and all compounds were individually tuned using ESI^-.

MS conditions for multiple reaction monitoring (MRM) were determined by examining fragments produced by the individual

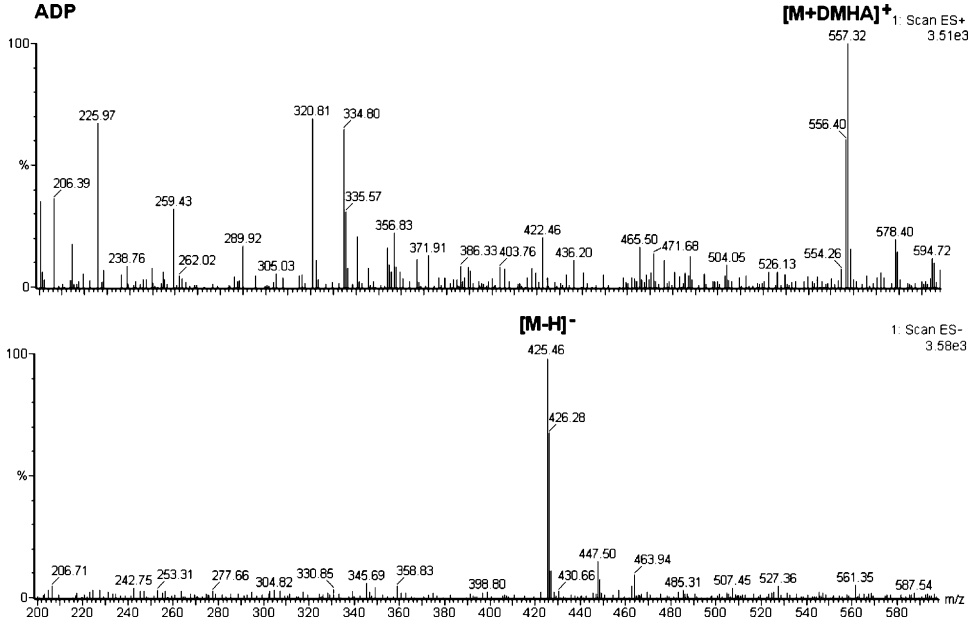

Fig. 3. Full scan spectra observed for ADP with (a) positive and (b) negative electrospray modes carried out at cone voltage of 40 V, source temperature 125 °C, with nitrogen drying gas and nebulising gas. The most abundant ions observed are $[M-H]^-$ 426 and $[M+DMHA]^+$ 557, in negative and positive mode, respectively.

Table 1
Table showing parameters of MRM scan derived for specific detection of different nucleotides

	Retention time (min)	Precursor ion $[M-H]^-$ (m/z)	Most prominent product ions (m/z)	Selected product ion (m/z)	Cone voltage (V)	Collision energy (V)
Adenine	4.9	133.86	91.97, 106.69	106.69	36	18
Adenosine	9.6	266.02	133.99	133.99	36	20
CMP	12.3	321.98	78.9, 97.01	78.9	37	23
UMP	13.2	322.98	78.87, 96.97	96.97	35	25
cAMP	17.0	327.95	133.62	133.62	41	26
cGMP	14.1	343.93	149.64	149.64	37	24
AMP	16.8	345.94	79.05, 96.75, 134.01	96.75	40	26
GMP	13.7	361.98	79.07, 96.89, 210.73	210.73	41	23
CDP	18.5	401.93	158.51	158.51	40	25
UDP	18.7	402.94	158.63	158.63	37	26
ADP	20.1	425.96	158.58, 327.55	327.55	43	18
GDP	19.3	442.01	158.71, 343.88	343.88	40	20
CTP	21.8	481.89	158.51, 383.74	383.74	38	24
UTP	22.0	482.9	159.06, 384.74	384.74	38	22
FMN	23.8	455.25	255.13	255.13	38	23
ATP	22.6	505.93	158.63, 407.75	407.75	39	24
GTP	22.9	521.98	158.87, 423.72	423.72	36	24
cADPR	9.8	540.04	407.6	407.6	36	37
NAD	10.7	662.28	539.6, 408.06	539.6	40	18
NADH	15.9	663.83	407.66	407.66	43	25
NADP	19.8	742.1	619.51, 408.06	619.51	40	15
NAADP	22.7	743.08	408.10, 620.10	620.10	36	22
NADPH	22.8	744.12	408.01, 663.94	663.94	40	33
FAD	23.6	784.14	408.29	408.29	38	32

compounds using a collision energy of 20 eV to produce spectra from which the dominant product ions could be chosen. Once the dominant product ion was determined the collision energy was optimised for the production of the selected ion. This process helped distinguish between some of the nucleotides that were only one atomic mass unit (amu) apart but which could be readily distinguished by selecting appropriate m/z ions from the product ion spectra. Optimised collision energies and cone voltages determined for each nucleotide are shown in Table 1. A common fragmentation pattern was observed for most of the nucleotides involving the production of m/z 79 and m/z 97, due to the loss of a phosphate group from the nucleotides, and further fragmentation with the di- and tri-phosphates resulted in the loss of further phosphate groups. Using the optimised LC–MS/MS approach all 24 nucleotides and related phosphorylated compounds were detected simultaneously in a 10 μM standard solution (Fig. 4).

3.3. Development of extraction methodology

Extractions of CHO cells with a variety of solvents and acids were carried out to investigate the effects on the recovery of nucleotides. Due to the widespread successful use of organic solvents as extraction solvents for LC–MS methods [21,32,33] and their known ability to precipitate proteins [34] the first extractions to be evaluated involved organic solvents. Methanol offered the most efficient extraction with an average recovery of 53% whereas ethanol and acetonitrile demonstrated much lower recoveries of 8 and 11%, respectively. Anomalous results were observed for the extractions involving 20% water; possibly due to either the solvent's inability to immediately stop cellular processes on contact, or to the difficulties in integrating the relevant peaks due to unwanted extracted co-interfering compounds. The chromatography was affected negatively in some cases by the extracts and very broad, split chromatographic peaks were produced. This was particularly evident with the di- and triphosphates when water was included in the extraction solvent. This chromatographic effect resulted in the loss of separation between the diphosphates and triphosphates so cross talk between the MRM channels became a problem with tandem MS detection. These problems in chromatographic resolutions resulted in the inability to determine the recovery of diphosphates and triphosphates standards from samples. The addition of the hexane extraction stage after the initial solvent extraction of the CHO cell extracts improved sample clean-up peak shapes and resolved many of the problems discussed above which we attributed to the removal of phosphate-containing lipids from the extract (Table 2).

Acid extractions with perchloric acid, formic acid and formic acid in methanol as extraction solvents were also evaluated. The perchloric acid extract, despite being reported for similar applications [13,16,19,35] was not compatible with LC–MS because of the presence of a precipitate when evaporating the neutralised sam-

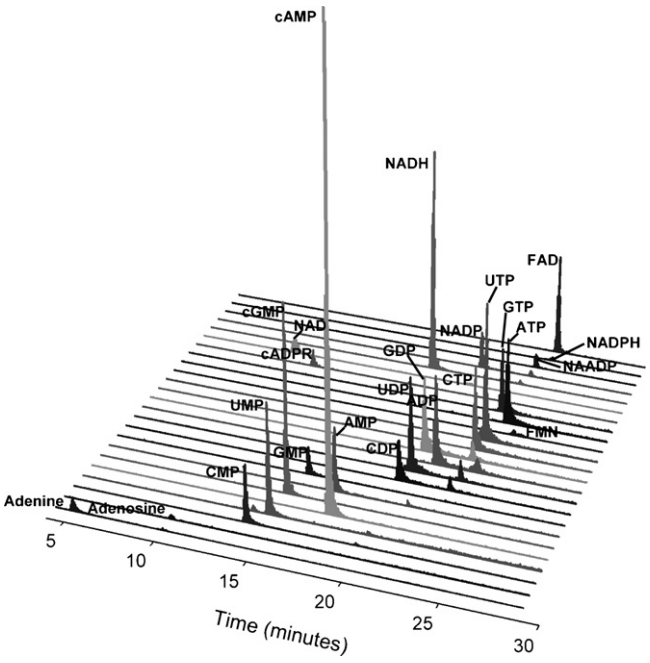

Fig. 4. Results from LC–MS–MS analysis using MRM scan (dwell 0.2 s) showing the simultaneous detection of 24 nucleotides in standard solution 10 μM.

Table 2
Results showing percentage recoveries for different acid extractions

	Extraction solvent (recovery%)			
	MeOH/hexane with sonication	MeOH/hexane without sonication	Formic acid in MeOH with sonication	Formic acid in MeOH without sonication
cAMP-BR	71.3	62.6	104.0	103.4
Adenosine	70.8	61.7	70.3	53.5
cGMP	61.4	54.2	66.9	76.6
cAMP	64.4	53.9	95.0	97.5
CMP	79.3	60.4	95.0	92.4
UMP	20.7	30.0	101.4	50.9
AMP	25.1	25.0	104.2	97.0
GMP	47.8	36.7	95.9	87.6
CDP	18.9	17.6	59.0	52.9
UDP	51.2	48.1	67.3	38.0
ADP	62.3	33.4	105.4	80.0
GDP	16.6	18.1	60.7	31.5
CTP	17.4	19.4	65.3	22.1
UTP	46.9	44.5	68.9	12.5
ATP	43.4	45.3	79.2	59.3
GTP	12.2	13.7	58.0	47.3
NAD	86.9	82.3	89.8	85.0
NADP	48.2	29.3	48.0	42.3
NAADP	53.4	28.6	53.4	28.6
FAD	67.1	54.0	7.7	9.2
FMN	89.3	77.4	9.2	93.0

Data shown represents 1 replicate from 3. Results from MRM scan (dwell 0.2 s) showing the simultaneous detection of 24 nucleotides cell extracts corrected for endogenous levels with blanks.

ples. Highly variable recoveries were observed, sometimes greater than 100% or even negative values, when formic acid alone was used as the extraction solvent (data not shown). These results suggested that cellular processes were not being immediately stopped by the addition of the formic acid but were being disrupted in some way leading to erroneous data, and hence this approach was deemed unusable. Sonication of the CHO cells in the well plates also generally improved recovery, possibly linked to improved disruption of the cellular membranes so more nucleotides were released (data not shown). However, the use of sonication was disregarded due to logistical problems of keeping the plates cool whilst being sonicated and difficulties in preventing water contaminating the samples.

Based on our investigations, the addition of methanol precooled to −20 °C followed by the hexane extraction was found to be the best extraction technique to be used. The method is simple, readily compatible with mass spectrometry and the removal of lipid soluble compounds with hexane also helped to improve peak shape reducing contaminants introduced into the mass spectrometer. Comparable extraction methods have been used for similar studies; Fung et al. [20] also use a methanol/water extraction method to lyse peripheral blood mononuclear cells and Cai et al. [13] use methanol followed by chloroform in a similar way to hexane for lipid removal, to extract metabolites from *Bacillus subtilus*.

3.4. Validation of the method

The validation data for the method is shown in Table 3. The results confirm that the method demonstrates reasonable recoveries of the analytes and were sufficiently linear, sensitive, precise and accurate for application to the measurement of these analytes in CHO cells. The intra-day precision was within an R.S.D.% of 1.9–16.4% and the inter-day was between 2.7 and 20.7%. The linearity of the method was confirmed by analysis of nine-point calibration standards over the range 0.5–100 μM.

The blank incubation matrix for CHO cell culture showed no interfering peaks for any of the analytes when examined by the LC–MS/MS method, confirming that the method was sufficiently selective to exclude any matrix effects. The majority of analytes showed little or no ion suppression effects but a potential problem was observed with ATP which had signals of 21 and 41%, respectively, of the expected values at the 1 and 10 μM concentrations. This ion suppression was not apparent at the higher 50 μM added concentration of ATP concentration. This result may have been due to the exceptionally high endogenous concentration of ATP (over 200 μM) making it difficult to measure the ion suppression effects of ATP at the much lower added concentrations of 1 and 10 μM. The lower limits of detection (S/N = 3:1) of the method for each analyte are shown in Table 3. The sensitivity achieved with the method is notably better than HPLC based methods with UV detection as expected. Previous HPLC-UV methods applied to nucleotide measurement in cell culture systems have detection limits in the 10–100 pmol range [6,7]. The assay method sensitivities we report (0.5–10 pmol) here are similar to those in other LC–MS methods [13,18,23] but the number of nucleotides analysed in our method are larger than previous other fully validated LC–MS methods. However, many methods that have been applied to biological samples lack a rigorous and comprehensive validation procedure [12,13,15,16,18].

3.5. Cell volume analysis

Using the confocal microscopy technique the average volume of nine CHO cells was found to be 4 ± 0.8 pl. The average number of cells per well was found to be $4.018 \times 10^6 \pm 3.52 \times 10^5$. This gave a total mean cell volume of 16.072 μl/well. This cell volume allows the conversion of quantitative measurements into real cellular concentrations of the nucleotides, often not included in other methods. The cell volumes we report for CHO cells agree closely with other mammalian cell volumes reported, for example, Conlon and Raff [36] found the cell volume of Schwann cells to be approximately 1.6 pl and Allansson et al. [37] found astroglial cells to have an approximate volume of 2 pl.

3.6. Profiling of intracellular nucleotides in CHO cells

The developed method was then applied to profile endogenous levels of intracellular nucleotides in cultured CHO cells under

Table 3
Precision, accuracy, recovery and linearity of the method at low (L) 1 μM, medium (M) 10 μM and high (H) 50 μM concentrations

	LOD (pmol on column) with 10 μl injection	Intra-day, $n = 6$									Inter-day, $n = 6$								
		Recovery (%)			Precision (R.S.D.%)			Accuracy (%)			R^2			Precision (R.S.D.%)			Accuracy (%)		
		L	M	H	L	M	H	L	M	H	L	M	H	L	M	H	L	M	H
Adenine	5	81	79	72	11.2	5.4	7.5	104.5	111.3	102.5	0.998	0.986	0.977	13.2	14.0	8.5	92.3	92.3	95.3
Adenosine	10	75	78	70	8.1	1.9	8.1	105.4	114.0	97.7	0.994	0.989	0.988	12.3	15.9	9.5	88.8	88.9	93.8
CMP	5	74	77	76	11.9	7.2	4.7	107.1	109.2	101.8	0.999	0.988	0.990	20.7	11.3	8.3	120.2	113.6	91.2
UMP	5	72	75	72	11.9	7.2	4.7	107.1	109.2	101.8	0.969	0.925	0.992	13.5	10.5	4.4	121.2	99.8	92.5
cAMP	0.25	76	75	76	5.7	2.9	3.7	114.0	114.6	102.0	0.999	0.993	0.995	11.4	6.9	2.7	99.3	100.2	85.6
cGMP	0.5	76	79	75	3.2	3.3	5.5	120.2	113.9	101.3	0.998	0.997	0.999	12.0	11.0	7.3	89.6	109.1	100.7
AMP	2.5	63	71	72	10.4	10.6	12.0	110.2	105.5	104.0	0.993	0.999	0.997	15.5	12.5	3.7	89.3	105.2	85.1
GMP	5	78	76	64	15.3	10.4	10.3	102.5	97.6	96.3	0.983	0.966	0.972	10.2	12.3	4.7	103.3	82.5	84.4
CDP	2.5	71	66	69	16.3	9.1	11.1	106.3	99.0	98.1	0.978	0.975	0.991	7.7	14.7	9.6	92.0	115.1	92.1
UDP	2.5	60	68	65	16.2	5.2	3.3	94.6	90.5	94.4	0.991	0.929	0.992	18.6	13.5	7.2	96.3	109.7	88.4
ADP	2.5	62	56	64	11.9	7.5	4.9	93.4	94.1	96.8	0.974	0.997	0.992	14.5	13.0	8.1	102.8	88.3	91.7
GDP	2.5	65	59	62	16.4	5.3	6.1	89.8	91.5	94.3	0.981	0.985	0.947	15.9	5.2	9.2	110.1	99.9	88.2
CTP	2.5	70	73	66	1.9	3.9	8.2	86.8	87.2	96.7	0.978	0.923	0.981	18.0	8.4	6.8	86.8	107.9	81.8
UTP	2.5	64	79	64	3.6	15.2	13.7	83.3	99.2	95.5	0.989	0.986	0.982	8.8	13.6	11.6	110.7	98.2	84.4
FMN	5	65	65	59	9.6	3.7	6.4	105.6	114.0	103.5	0.983	0.997	0.985	18.7	12.6	9.3	108.7	83.8	85.4
ATP	2.5	74	69	53	12.8	2.6	9.2	90.5	87.5	98.2	0.974	0.931	0.992	15.4	9.1	8.9	115.9	82.5	85.0
GTP	2.5	64	68	58	12.8	8.6	12.8	96.4	92.5	98.5	0.965	0.928	0.981	14.3	9.1	5.9	113.3	83.0	83.0
NAD	5	72	71	67	14.5	1.4	7.7	96.4	86.2	97.8	0.989	0.952	0.955	16.3	10.2	7.8	95.0	110.0	93.4
NADH	5	73	73	77	12.1	8.9	9.2	89.0	111.2	92.2	0.978	0.985	0.990	13.1	8.5	9.7	86.0	116.1	89.3
NADP	2.5	56	60	57	12.2	8.3	4.8	93.0	98.3	104.1	0.982	0.978	0.983	15.0	13.6	11.5	104.1	95.1	82.3
NAADP	5	68	65	59	13.1	10.4	11.5	102.2	101.6	97.8	0.946	0.990	0.986	7.8	9.7	3.3	91.8	109.2	86.1
NADPH	5	73	75	68	11.3	9.1	7.0	101.9	93.2	96.6	0.990	0.978	0.987	6.7	8.1	9.4	97.3	94.5	93.8
FAD	5	72	70	69	12.9	6.1	11.3	103.7	111.8	100.8	0.968	0.963	0.977	12.6	15.0	6.3	103.7	92.8	92.4

Results using MRM scan (dwell 0.2 s) showing the simultaneous detection of 24 nucleotides cell extracts.

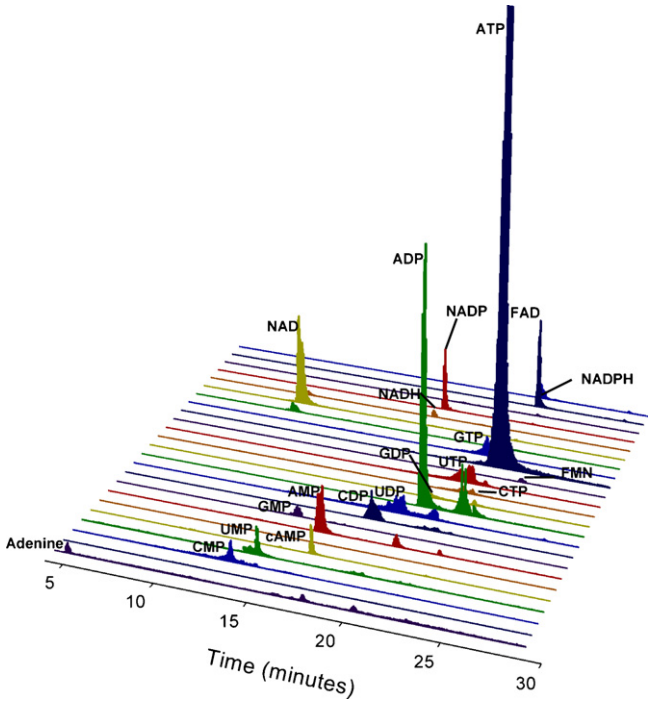

Fig. 5. Results of MRM scan (dwell 0.2 s) for biological sample in control conditions.

standard culture conditions and after pharmacological intervention with isoprenaline to act on the β_2 adrenoceptor to increase cAMP levels and IBMX a phosphodiesterase inhibitor to prevent the cAMP breakdown. The number of cells in each well was 4×10^6 making a volume of around 16 μl, i.e. a weight of approximately 16 mg per sample. Despite the very small volume most nucleotides and related compounds could be easily detected in the extract from a single well (Fig. 5) with the exception of adenine, cADPR, cGMP and NAADP which were below detection limits due to either low or non-existent cellular concentrations, and adenosine which had a LOD significantly higher than the other compounds due to poor ionisation at the MS source.

The intracellular nucleotide concentrations measured in the CHO cell control group (Table 4) compare reasonably well with other data available on nucleotides in mammalian cells [4,7,31,38]. These papers each quantify a limited range of nucleotides but are useful to compare with what our method can achieve from a single small sample and analytical run. The highest concentration found in these papers was that of ATP, found to be around in the range of 0.82–6.3 nmol/10^6 cells and, as can be seen in Table 4, we also found ATP to be the nucleotide of highest concentration in CHO cells at 0.89 nmol/10^6 cells. The other triphosphates were present in lower but in the same magnitude concentrations from 0.02 to 3.2 nmol/10^6 cells, and were all easily detected in our method at 0.05–0.13 nmol/10^6 cells. The diphosphate levels were variable with CDP being the lowest and often below the limit of detection as also found by Cichna et al. [7]. Our results determined UDP and GDP at 0.01 nmol/10^6 cells and ADP somewhat higher at 0.41 nmol/10^6 cells whereas UDP and GDP have been previously reported at higher concentrations of around 0.28 nmol/10^6 cells [7] but ADP at 0.6–1.53 nmol/10^6 cells is comparable. The monophosphates had the lowest concentrations (0–0.03 nmol/10^6 cells) with CMP often not detectable, this being in agreement with previous investigations [7]. The relative levels of some of the adensine nucleotides obtained here, however, suggest that there maybe a problem with either cell viability or possibly the speed of action of the extraction process on stopping intracellular enzyme activity.

Table 4
Concentration of nucleotides in CHO cells and effects of isoprenaline, IBMX and isoprenaline and IBMX

	Control group nucleotide concentration (μM)	S.D.	nmol/million cells	Isoprenaline treated group nucleotide concentration (μM)	S.D.	IBMX treated group nucleotide concentration (μM)	S.D.	Isoprenaline + IBMX treated group nucleotide concentration (μM)	S.D.
cAMP	0.63	0.09	0.00	14.2	0.80	3.80	0.80	52.5	8.02
CMP	6.68	0.52	0.03	4.00	0.71	5.00	0.63	3.70	0.50
UMP	8.30	0.81	0.03	8.20	1.56	7.30	0.82	5.80	1.30
AMP	12.7	1.39	0.05	15.2	1.12	12.6	1.80	8.30	1.32
GMP	0.98	0.20	0.00	0.90	0.13	0.90	0.17	0.70	0.10
CDP	0.10	0.00	0.00	0.20	0.00	0.20	0.00	0.20	0.00
UDP	3.40	0.31	0.01	2.10	0.30	2.20	0.10	1.20	0.20
ADP	102	16.3	0.41	96.4	5.40	99.5	11.3	79.4	11.8
GDP	2.88	0.51	0.01	2.80	0.22	3.40	0.50	1.50	0.20
CTP	13.5	1.53	0.05	12.0	2.40	14.4	1.91	7.50	1.11
UTP	33.7	3.60	0.13	22.0	2.56	27.9	3.13	13.5	1.92
ATP	223	21.8	0.89	136	16.3	144	14.0	72.6	8.13
GTP	18.9	2.30	0.08	7.80	1.30	9.90	1.66	3.70	0.60
FAD	3.45	0.39	0.01	3.10	0.30	3.20	0.53	2.80	0.56
FMN	0.43	0.10	0.00	0.30	0.02	0.40	0.00	0.30	0.03
NAD	70.7	12.2	0.28	57.2	6.05	48.5	3.80	37.3	5.10
NADP	5.05	0.90	0.02	4.90	0.80	4.60	0.99	2.40	0.30
NADPH	75.2	13.5	0.30	60.6	13.0	56.20	5.10	45.9	4.06

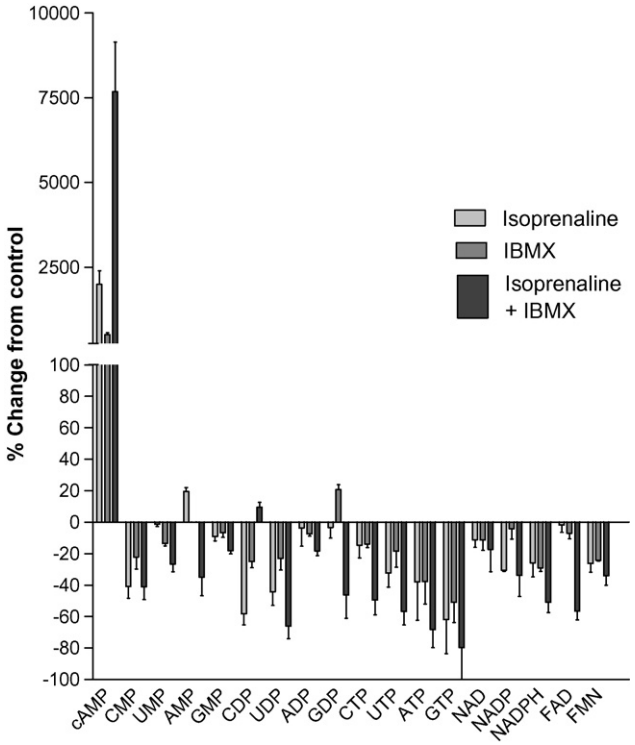

Fig. 6. Relative changes in integrated peak areas of different nucleotides from the control group of the different drug treated cell groups.

In a healthy viable cell the expected ratio is in the order of 10:1 ATP to ADP whereas in the control group here this ratio is lower at a just over 2:1. In general though, the results obtained appear in good agreement with accepted literature values suggesting that the simultaneous detection of the nucleotides does not compromise accuracy of the method.

In the isoprenaline treated CHO cells there was a large increase in cAMP levels in compared with the control group (Fig. 6). Isoprenaline is a sympathomimetic acting drug which acts almost exclusively on the β-adrenoceptors stimulating the observed increase in cAMP. With the addition of IBMX to the CHO cells only a small increase occurred but when combined with isoprenaline the measured cAMP concentrations increased hugely as expected, since the enzymic degradation of cAMP by phosphodiesterases is inhibited by IBMX.

The use of these drugs for pharmacological intervention is common [39] and their effects on the cells in relation to cAMP levels is well established. It is however often assumed that the effect on the second messenger cAMP is the only effect whereas the experiments carried out in this paper suggest that there may be more global effects on the nucleotide pool. Most of the nucleotides measured showed marked decreases in concentration after undergoing either drug treatment, but the decreases were greatest after the IBMX/isoprenaline combined treatment (Fig. 6). This suggests that the drugs are having a negative affect on the overall cell's function, with all the cells resources being channelled into the production of cAMP. The ADP:ATP ratio in the drug treated groups also shows a decrease to 1.4 in the isoprenaline or IBMX treated cells to as low as 0.9 in the cells exposed to the combined drug treatments. These effects may have implications in experiments involving these drugs as their effects are possible not as targeted as previously thought and may have effects on cell viability.

4. Conclusions

It has been confirmed that complex mixtures of nucleotides can be separated and detected using reversed-phase HPLC coupled to ESI^- mass spectrometry with a methanol/water gradient separation and the use of the volatile ion-pairing agent DMHA. The method has also been shown to have sufficient sensitivity and selectivity to be applied to the quantification of the nucleotide pool in small amounts (approximately 16 mg) of cultured mammalian cells, thus enabling nucleotide determination in single wells. The sensitivity of the method is comparable or better than other LC–MS methods [16–18] and the number of analytes is significantly greater than reported in many previous LC–MS methods [12–14,16–18,20]. The use of such a method has the potential to give new insight into the actions of pharmacological agents on nucleotide homeostasis *in vitro* with the ability to simultaneously measure an array of nucleotides previously not achievable.

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