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NEW APPLICATIONS OF CAPILLARY ELECTROPHORESIS COUPLED WITH MASS SPECTROMETRY FOR THE EVALUATION OF ENDOGENOUS METABOLITES OF CLINICAL INTEREST IN URINE

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SUMMARY

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1. General aim

One of current fields in Analytical Chemistry for application in biomedical areas is the establishment of rapid and efficient methods of analysis for the determination of endogenous metabolites of clinical interest in biological fluids.

Free and modified nucleosides and nucleobases lie within this field; they are compounds that are excreted naturally in urine as products of transfer RNA (tRNA) regeneration and of DNA repair as the result of damage due to oxidative stress. Their levels in urine increase anomalously in certain pathologies, such as tumour processes and neurodegenerative diseases, such that these substances are of great interest as biomarkers in clinical chemistry. Reports have been made of the potential usefulness of free and modified nucleosides as biological markers in diseases as diverse as rheumatoid arthritis, Alzheimer's disease and AIDS, but mainly as early markers of many different types of cancer.

Some authors have also reported studies relating the urinary levels of modified nucleosides to other benign diseases and different lifestyles, such as smoking, fatigue, stress, poor nutrition, exposure to toxic compounds, etc.

Accordingly, most of these compounds lack specificity, such that they cannot be used as sole diagnostic tests. By contrast, they are useful for the early diagnosis of certain diseases, in the control of the efficacy of a given treatment or specific therapy and in the monitoring of post–operative recurrences.

The aim of this work was to develop rapid and efficient methodologies that could be used for the separation, identification and quantification of a broad set of free and modified nucleosides in biological fluids. Urine was chosen as the matrix because it is readily available and its collection is non–invasive.

As analytical technique we used capillary electrophoresis coupled with mass spectrometry (CE–MS), which combines the advantages inherent to capillary electrophoresis as a high performance separation technique and the ability of mass spectrometry to identify analytes faithfully. Moreover, capillary electrophoresis is highly appropriate for application in the analysis of biological samples because it is compatible with aqueous media and is suitable for the separation of highly polar compounds, both ionic and ionizable. These requirements match most of the metabolites found in biological fluids such as urine.

For its application to a complex matrix such as urine it was necessary to develop extraction/cleaning/preconcentration procedures in order to favour analyte detection and preserve the instrumental set–up. Here we studied the application of

a widely used technique (solid phase extraction, SPE) and also a more recently developed one, based on the use of a restricted access material (RAM) owing to the capacity of this type of material to exclude macromolecules thanks to a molecular exclusion mechanism.

A final aim was to check the applicability of the methodologies developed in the analysis of urine samples from healthy donors, smokers and non–smokers. This part of the study aimed at confirming whether tobacco consumption affects the levels of these nucleosides in urine, with respect to the reference levels of healthy non–smokers. This could help to determine whether this habit might exert possible interference in the use of the compounds studied as markers of severe diseases.

To conclude, a search for other metabolites possibly associated with tobacco consumption is described. To accomplish this, a mass spectrometry detector with high accuracy and resolution, such as a Time–of–Flight (TOF) detector, was used for the analysis of urine samples from smokers and non–smokers.

2. Results and discussion

2.1. DETERMINATION OF NUCLEOSIDES BY CE–ESI–MS IN POSITIVE MODE.

The work reported here consisted of developing a method based on capillary electrophoresis coupled with mass spectrometry for the separation, identification and quantification of a set of nucleosides, nucleobases and methylated derivatives in urine samples.

This chapter can be divided into three parts:

- Study of electrophoretic separation.
- Determination in urine samples.
- Development of a procedure for extraction–preconcentration.

From the results obtained in each of the above the following conclusions can be drawn:

Study of electrophoretic separation

Using capillary zone electrophoresis (CZE) we studied the electrophoretic behaviour of free and modified nucleosides with different separation media: acid and base.

For this part of the study we used an initial set of 13 analytes, among which there were nucleosides such as adenosine, guanosine, inosine and uridine; nucleobases such as guanine; methylated derivatives such as 7–methylguanine, 1–methylguanine, 9–methylguanine, 1–methyladenosine, 7–methylguanosine and 5–methyluridine, and hydroxylated derivatives such as 8–hydroxy–2'–deoxyguanosine.

In acid medium, the analytes susceptible to migrate electrophoretically are:

Guanine (Gua)	1–methyladenosine (1mA)
7–methylguanine (7mGua)	Cytidine (Cyt)
1–methylguanine (1mGua)	7–methylguanosine (7mG)
9–methylguanine (9mGua)	Guanosine (G)
Adenosine (A)	

Whereas in alkaline medium it is possible to separate the following:

Guanine (Gua)	7–methylguanosine (7mG)
7–methylguanine (7mGua)	2'–deoxyguanosine (2dG)
1–methylguanine (1mGua)	Guanosine (G)
9–methylguanine (9mGua)	Inosine (INN)
Adenosine (A)	5–methyluridine (5mU)
1–methyladenosine (1mA)	8–hydroxy–2'–deoxyguanosine (8OH2dG)
Cytidine (Cyt)	

Later studies focused on developing a method using capillary zone electrophoresis in acid medium with the analytes susceptible to migrating under these conditions.

Optimization of electrophoretic separation in this acid medium involves the following conditions:

- ⇨ Electrophoretic separation medium (background electrolyte, BGE): 100 mM formic acid–ammonium formate, pH 2.5.
- ⇨ Voltage applied: 20 kV up to 12 min; 25 kV from 12 to 15 min, and 30 kV for 15 min and longer.
- ⇨ Hydrodynamic injection: 8 sec at 50 mbar.
- ⇨ MS detection:
  - Quadrupole analyzer.
  - Electrospray interface (ESI), positive mode; capillary voltage, +3500 V; additional liquid flow rate, 0.78 μL min^{-1}; nebulizer gas pressure, 10 psi; nebulizer gas flow rate, 6 L min^{-1} and nebulizer gas temperature, 150 ºC.
  - Additional liquid: 1:1 (v/v) UHQ water:isopropanol with 7.5 mM acetic acid.

Determination in urine samples

Application to urine samples revealed the different concentration levels at which the analytes are found naturally.

In the case of the analytes present at high concentrations, 7mGua, 1mA and A, a quantification method based on calibration in synthetic urine was proposed as an alternative to the standard additions method since it provided similar results. These results were confirmed with LC–MS/MS.

For the analytes present at medium and low concentrations, Gua, 1mGua, 9mGua, Cyt, 7mG and G, a preconcentration procedure based on SPE was developed. This is described below.

Development of a preconcentration procedure

We describe a procedure for the extraction–preconcentration of the analytes studied in urine samples based on the use of SPE with a polymeric sorbent, ENV+, which is a hydroxylated copolymer of polystyrene–divinylbenzene.

A volume of urine of 2.0 mL was used in the proposed procedure. Analyte elution was performed with 2.0 mL of 90:10 (v/v) ACN:NH_3, which was evaporated off and the residue was redissolved in 500 μL of UHQ water with 0.1% (v/v) formic acid prior to injection into the CE system. The recoveries obtained for the analytes studied ranged between 68% and 100% in urine samples.

In general, it may be concluded that the methodology developed in this work, based on the coupling of capillary zone electrophoresis to mass spectrometry (CE–ESI–MS) is rapid and robust, and that it is appropriate for the determination of modified nucleosides and nucleobases.

Published results:

- "Capillary electrophoresis–mass spectrometry for direct determination of urinary modified nucleosides. Evaluation of synthetic urine as a surrogate matrix for quantitative analysis", J. Chromatogr. B 942–943 (2013) 21–30.
- "Development of a procedure for the isolation and enrichment of modified nucleosides and nucleobases from urine prior to their determination by capillary electrophoresis–mass spectrometry", J. Pharm. Biomed. Anal. 88 (2014) 489– 496.

2.2. DETERMINATION OF NUCLEOSIDES BY CE–ESI–MS IN NEGATIVE MODE.

We next developed a methodology based on CE–MS for the separation of modified nucleosides susceptible to migrating electrophoretically as anions in basic medium. In this case, later detection by MS was performed by electrospray ionization in negative mode, ESI(-).

In this part of the study we used a set of 5 analytes, among which the following were included: the hydroxylated derivatives 8–hydroxyguanosine (8OHG) and 8–hydroxy–2'–deoxyguanosine (8OH2dG), which are metabolites associated with processes of oxidative stress, together with nucleosides such as inosine (INN) and other derivatives such as 5–methyluridine (5mU) and 2'–deoxyguanosine (2dG).

From the results obtained from this second method, the following conclusions can be drawn:

Study of electrophoretic separation.

Electrophoretic separation using capillaries with different internal diameters –50, 75 and 100 μm– revealed that 50 μm was the most suitable diameter for the separation of the analytes studied since it provided the best resolution within a given time of analysis.

Optimization of electrophoretic separation in basic medium and with this capillary diameter (50 μm) involved the following conditions:

- ⇨ Electrophoretic separation medium (background electrolyte, BGE): 30 mM ammonium formate–ammonia, pH 11.0.
- ⇨ Voltage applied: 30 kV.
- ⇨ Hydrodynamic injection: 30 sec at 50 mbar in a 1:10 (v/v) BGE:UHQ water injection medium.
- ⇨ MS detection:
  - Quadrupole analyzer.
  - Electrospray interface (ESI), negative mode; capillary voltage, -3500 V; additional liquid flow rate, 0.78 μL min^{-1}; nebulizer gas pressure, 6 psi; nebulizer gas flow, 7 L min^{-1} and nebulizer gas temperature, 350 ºC.
  - Additional liquid: 1:1 (v /v) UHQ water:isopropanol.

Determination in urine samples

Application to urine samples revealed the low concentration levels of the analytes studied. The development of a previous sample treatment step was based on SPE with polymeric sorbents and on the use of a restricted access material (RAM).

Solid phase extraction (SPE) as a previous step.

We report a procedure for the extraction of the analytes studied in urine samples that is based on the use of a polymeric sorbent, ENV+, a hydroxylated copolymer of polystyrene–divinylbenzene.

The proposed procedure employed a 5.0 mL volume of urine diluted 1:1 (v/v) with UHQ water. Analyte elution was accomplished with 2.0 mL of a 90:10 (v/v) MeOH:NH_3, which was evaporated off and the residue was redissolved in 1.0 mL of 1:10 (v/v) BGE:UHQ water before injection into the CE–ESI–MS system. In this case, the injection time was reduced from 30 to 10 sec.

The optimized conditions in this part of the study were satisfactory for the separation and quantification of some of the analytes studied, 8OHG and 8OH2dG, although they did not provide acceptable results for all of them. Recoveries ranged between 69 and 93%.

Use of a restricted access material (RAM) as a previous step

We describe a procedure for the extraction of the analytes studied in urine samples based on the use of a restricted access material (RAM) packed with a hydrophilic copolymer containing N–vinylacetamide, with a size of 10mm x 4.0mm.

In the proposed procedure we used a volume of 500 μL of urine diluted 1:1 (v/v) in UHQ water. Elution of the analytes retained in the RAM was conducted with ACN:UHQ water at a proportion of 70:30 (v/v), with a flow rate of 0.5 mL min^{-1} over 4 min. The eluted fraction was evaporated off and the residue was

redissolved in 250 μL of 30 mM BGE spiked with the internal standard prior to injection into the CE–ESI–MS system.

Analysis of nucleosides in urine samples as a function of smoking or non–smoking.

The methodology developed –RAM/CE–ESI(-)MS– was applied to the study of the analytes of interest in urine samples from healthy donors who smoked. We were interested in determining whether there were any significant differences in the concentrations of these analytes in urine samples from smokers as compared with samples taken from non–smokers.

The aim underlying this was to establish a possible relationship between the levels of these modified nucleosides and tobacco consumption in healthy individuals. Healthy individuals were selected in order to avoid the influence of possible diseases on the levels of these metabolites and hence establish the specific effect of the smoking habit.

In 42 urine samples from healthy individuals (21 smokers and 21 non–smokers, balanced according to age and gender), low concentration levels of these compounds were observed in all of them. The results obtained with the use of the method developed (RAM/CE–ESI(-)MS) were validated by LC–MS/MS).

These results showed that there were no significant differences in the concentrations of these analytes between samples from smokers and from non–smokers. We also failed to detect significant differences in the concentrations of the analytes among the smokers as a function of their age and gender.

At least in light the above studies, there is no experimental evidence to be able to conclude that the concentration of the nucleosides studied here in urine (among them the 8OH2dG and 8OHG derivatives) is higher in smokers than in non–smokers. Accordingly, in healthy individuals tobacco consumption does not seem to alter the urinary levels of these compounds.

Nevertheless, it should not be forgotten that the sampling carried out here was only done in healthy individuals (middle–aged and young people), suggesting that it would be necessary to conduct further studies, broadening the sample

population to older individuals and to individuals already affected by a disease, all of them included in the category of smokers and non–smokers.

Finally, it should be stressed that the aim of this study was to demonstrate a possible application of the methodology proposed as an analytical tool for use in clinical practice, with no intention of further entering this field itself since it is evidently beyond our reach as chemists.

Published results:

- "Determination of hydroxylated nucleosides in human urine by capillary electrophoresis with mass spectrometric detection." Communication presented at 20th International Symposium on Electro– and Liquid Phase Separation Techniques, ITP 2013. Pending publication.

2.3. APPLICATION TO THE STUDY OF METABOLITES ASSOCIATED WITH TOBACCO CONSUMPTION.

In the previous section, we reported the development of a method based on CE–ESI(-)MS for the detection and quantification of some modified nucleosides of interest, among them the hydroxylated derivatives 8OHG and 8OH2dG. Application of the proposed method for the analysis of urine samples from smokers and non–smokers, all of them healthy, did not reveal significant differences as regards the levels of this group of compounds.

Accordingly, it was decided to broaden the study to other possible modified nucleosides that had not been included previously and in general to search for metabolites related to tobacco consumption that might show significant differences between the two groups of samples.

A study was conducted to obtain the metabolic fingerprint of the urine samples through the use of a Time–of–Flight (TOF) mass analyzer coupled with a liquid chromatograph (LC–TOF). In this study, the sample treatment step with the RAM described above was maintained, but the parameters affecting chromatographic separation in reverse phase mode were optimized.

The approach of this study is based on the experience acquired by the PhD student during her research stay, developed as part of their pre–doctoral training, at King's College London (Department of Pharmaceutical Chemistry). [Nanotoxicology. 2015 Feb; 9 (1): 106–115].

From the results, the following conclusions can be drawn:

• From accurate mass analysis the identity of the 5 nucleosides under study in the urine samples was confirmed (metabolite target analysis). The lower sensitivity of TOF made their quantification almost impossible in most of the samples owing to the low concentration at which they are present in urine, both in smokers and in non–smokers.

• The second analysis was an analysis of the metabolic fingerprint whose aim was to search for possible significant differences in the metabolic profiles of the samples from smokers and non–smokers. From the study of these profiles, it would be feasible to identify other possible metabolites associated with tobacco consumption responsible for the differences between both sample sets. Models were generated with all possible combinations, bearing in mind variables such as gender,

age and the condition of being a smoker or not. Only those that compared the smokers with the non–smokers were valid, with no differences as regards gender or age.

From the results of this study it may be concluded that there were significant differences in the metabolic profiles of smokers and non–smokers. However, none of the metabolites responsible for such differences could be attributed a chemical structure related to the modified nucleosides according to the search carried out in the Human Metabolome Database (HMDB). Neither was it possible to identify them on the basis of compounds related to tobacco consumption such as derivatives of the nicotine metabolic pathway.

The results obtained in this section of the work indicated that in healthy individuals there is no experimental evidence that tobacco consumption is as additional factor in the increase in the levels of modified nucleosides in urine.

Again, it should be stressed that the sampling was limited, further and more specific studies being required to confirm the results.

Published results:

- "A metabolomic approach to the study of modified nucleosides in urine from healthy smoker and non–smoker." (in preparation).

3. General conclusions

The work described here consisted of the development of new methodologies based on capillary electrophoresis coupled with mass spectrometry (CE–MS) for the separation, identification and quantification of a set of nucleosides, nucleobases and some of their methylated and hydroxylated derivatives in urine samples. The work was completed with a study to search for and identify possible metabolites associated with tobacco consumption.

In the corresponding sections we have described the particular conclusions drawn for each step of these studies. Below we note the most relevant general conclusions.

DETERMINATION BY CE–ESI–MS.

1. We have established two new analytical methods based on CE coupled with MS (CE–MS) for the determination of nucleosides excreted in urine. In both methods, capillary zone electrophoresis (CZE) was used, either in acid medium, for the separation of compounds susceptible to generating cationic species, or in alkaline medium, for the separation of anionic species.

2. Detection via mass spectrometry was carried out with a simple quadrupole analyzer with a coaxial electrospray ionization source functioning in positive, ESI(+), or negative ESI(-) mode, depending on the possibility of ionization of the compounds studied.

APPLICATION TO URINE ANALYSIS.

3. Application to urine samples from healthy individuals revealed the different concentration levels at which each analyte was naturally present in the urine from the same individual and, furthermore, the great variability with which the same compound appeared among individuals.

4. For the analytes present at high concentrations a quantification method employing calibration in synthetic urine can be proposed as an alternative to the standard additions method. The results were confirmed with LC–MS/MS.

5. In the case of analytes present at intermediate or low levels (Gua, 1mGua, 9mGua, 7mG and G, in acid medium, and the hydroxylated derivatives in alkaline medium) we developed sample treatment steps based on SPE with polymeric sorbents and on the use of a restricted access material (RAM), facilitating the detection of the analytes, and at the same time preserving the instrumental set–up.

DETERMINATION OF MODIFIED NUCLEOSIDES IN URINE FROM SMOKERS AND NON-SMOKERS.

6. The RAM/CE–ESI(-)MS method developed for the determination of anionic species, such as the hydroxylated derivatives 8OHG and 8OH2dG, was applied to the study of these compounds in urine samples from healthy smoker and non–smoker donors in order to determine whether there were any significant differences in the concentrations of these analytes between the two groups.

7. The sampling was carried out on 42 urine samples from healthy volunteers, 21 of them smokers and the rest non–smokers. The groups were balanced as regards gender and age.

8. The experimental results, validated by means of LC–MS/MS, indicate that for the group of analytes studied there are no significant differences between the urine samples from smokers and non–smokers.

APPLICATION TO THE STUDY OF METABOLITES ASSOCIATED WITH TOBACCO CONSUMPTION.

9. The samples of urine from smokers and non–smokers were analyzed with a Time–of–Flight analyzer coupled with a liquid chromatograph (LC–TOF), using a RAM material as the previous clean–up step.

10. The analysis of target metabolites applied to the hydroxylated derivatives 8OHG and 8OH2dG, together with 5mU, 2dG and INN, allowed the identity of the 5 compounds studied to be determined via accurate mass analysis. However, the lower sensitivity of TOF hindered their quantification in most of the samples owing to the low concentration at which they are naturally present in urine, both that of smokers and that of non–smokers.

11. Study of the metabolic fingerprint of these urines samples from smokers and non–smokers allow the conclusion that both populations are significantly different but not as regards these endogenous compounds. Neither can they be discriminated on the basis of compounds derived from the nicotine metabolic pathway.

Overall, it may be concluded that the methodologies developed here, based on CE–ESI–MS, are rapid and robust. They are suitable for the determination of the modified nucleosides and nucleobases studied in urine samples.

The experimental results described here for the application of the methods to the analysis of urine from smokers and non–smokers do not point to any evidence that in healthy individuals tobacco consumption is an additional factor in the increase in the levels of these modified nucleosides in urine with respect to the reference values in healthy non–smoker individuals. However, it should not be overlooked that the sample size was small and the results should therefore be considered preliminary.

Finally, it is important to note, once again, that the aim of these studies was to demonstrate a possible field of application for the proposed methodologies as analytical tools, without any intention of becoming involved in clinical diagnosis, which is evidently not part of our knowledge and expertise as chemists.

4. PUBLISHED RESULTS

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Capillary electrophoresis–mass spectrometry for direct determination of urinary modified nucleosides. Evaluation of synthetic urine as a surrogate matrix for quantitative analysis

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ABSTRACT

This work describes the development of a fast and reliable method based on capillary zone electrophoresis coupled with electrospray ionization–mass spectrometry (CZE–ESI–MS) for the determination of modified nucleosides in untreated human urine. The target compounds were guanine, 1-methyl-guanine, 7-methyl-guanine, 9-methyl-guanine, adenosine, 1-methyl-adenosine, cytidine, guanosine, 7-methyl-guanosine. As internal standards, ribose-2-^{13}C-adenosine and 8-^{13}C-guanine were used. The CZE separation was carried out in acidic medium (pH 2.5). MS detection with a single quadrupole, with ESI operating in positive-ion mode, was optimized. For the analysis of urine samples, owing to the endogenous character of these analytes different quantification strategies were explored. The standard additions method, matrix-matched calibration in synthetic urine and calibration in pure aqueous medium were compared in order to evaluate the endogenous levels of these compounds in human urine. The results obtained showed that calibration in synthetic urine as a surrogate matrix was an appropriate alternative to the method of standard additions for the accurate quantitation of compounds such as guanine, 1-methyl-guanine, 7-methyl-guanine, adenosine, 1-methyl-adenosine and cytidine by CE–ESI–MS directly in the urine matrix; values in the range 0.1 μg/mL for cytidine and 6.4 μg/mL for 7mGua, as the lowest and the highest level, were found in untreated urine from healthy volunteers. These results were confirmed by LC–MS/MS detection. It can be concluded that the electrophoretic CZE–ESI–MS methodology offers a valid and reliable alternative for the determination of urinary nucleosides at naturally occurring levels in healthy individuals.

1. Introduction

The detection and quantification of certain endogenous metabolites such as free and modified nucleosides present in biological fluids (urine, blood and saliva) have aroused considerable interest in recent years owing to their usefulness as non-invasive diagnostic and/or follow-up methods for certain pathologies (tumoral processes, degenerative diseases. . .) and, in general, as a contribution to knowledge of the metabolic response of living organisms to physiopathological stimuli.

Free and modified nucleosides are degradation products of nucleic acids that are naturally present in human urine. This is due to a fundamental metabolic process called metabolic re-exchange of ribonucleic acid (RNA), during which the action of hydrolytic enzymes such as ribonucleases and phosphatases release normal and modified nucleosides during RNA regeneration, mainly transfer RNA (tRNA). Unmodified nucleosides undergo re-use and degradation, becoming transformed into uric acid (in the case of the analytes guanosine and adenosine), into β-alanine (in the case of cytidine and uridine) and β-aminoisobutyrate [1,2]. By contrast, modified nucleosides cannot be re-used or undergo later degradation, such that they circulate freely through the blood stream until they are eliminated in the urine. This natural metabolic process is enhanced in the presence of phenomena associated with cell ageing, carcinogenesis and neurodegeneration, and also with other diseases that course with strong metabolic disorders.

Modified nucleosides excreted in urine have been studied to examine their biomedical significance as possible biomarkers for cancer and other diseases (rheumatoid arthritis, encephalomyopathy, Alzheimer's and AIDS). It has also been suggested that the quantification of urinary levels of modified nucleosides might be useful for premature cancer diagnosis However, these biomarkers lack sufficient specificity for their use as single diagnostic tests since descriptions have been made of alterations of the urinary levels of

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modified nucleosides in cases of benign diseases and indulgence in certain life-style habits such as smoking, excessive sports activities, fatigue, stress, exposure to toxic compounds. . .). Accordingly, a highly active field of current research is devoted to assessing the possibility of using modified nucleosides to distinguish between cancer and other diseases. It is therefore important to stress that the clinical use of modified nucleosides and nucleobases is complementary to already known diagnostic methods. Another possible use of interest for these biomarkers is the follow-up of patients being treated for cancer, for monitoring the response to therapy, and in post-operative recurrences. If a marker or group of markers is available for a given type of cancer, it is much easier to check treatment efficacy by following the evolution of these compounds instead of repeating other much more costly tests.

Different analytical techniques have been reported for the determination of nucleosides, immunoassay [3–5] and, mainly, liquid chromatography with spectrophotometric [6–13] or MS detection [14,15] being the most widely employed. Application of capillary electrophoresis for the separation of modified nucleosides has also been reported [16–21] but in most cases the methods are based on MEKC procedures, which involve the use of surfactants in the separation medium. This hinders the application of MS detection coupled with CE.

Capillary electrophoresis (CE) is highly suitable for application in the analysis of biological samples since it is a technique that is compatible with aqueous media and is appropriate for the separation of highly polar analytes, both ionic and ionizable. These characteristics are seen in most of the metabolites found in biological fluids such as urine.

Capillary Electrophoresis offers a good alternative to chromatographic methods for the analysis of hydrophilic compounds. Capillary zone electrophoresis (CZE) has the advantage of separating charged analytes whereas reversed-phase LC separates analytes according to their interaction with the hydrophobic stationary phase. As a result, polar charged species are often not well separated by reversed-phase LC. Especially in these cases, CE represents a valid alternative to consider.

Other attractive features of CE are its general applicability to a broad range of analytes (from ions and small molecules to macromolecules), excellent separation efficiency, high speed of analysis, high versatility in terms of separation modes and, additionally, reduced sample and solvent consumption. Additionally, CE–MS coupling has the advantages inherent to capillary electrophoresis as a high-efficacy separation technique with the ability to identify compounds by mass spectrometric detection.

The aim of this work is to develop a fast and efficient analytical methodology based on CE–MS that permits quantitative assessment of the urinary levels of these compounds in untreated samples, and thus the method may be easily applied in clinical analysis for preventive uses or for follow-up in therapies.

The interest in urinary modified nucleosides as biomarkers for malignant diseases and, especially, their possible use for premature cancer diagnosis, has created the need for reliable analytical methods to quantify these compounds in human urine accurately.

Quantitative determination of modified nucleosides in urine is complicated by the endogenous presence of these analytes [22]. Another issue associated with quantitative bioanalytical methods based on MS detection is the so-called matrix effect, i.e., an alteration in the response due to the presence of coeluting compounds that may increase or reduce ionization of the analyte (ion enhancement/suppression) in the mass spectrometer source. Matrix-dependent signal suppression or enhancement represents a major drawback in quantitative bioanalysis based on MS detection. This issue has been widely discussed in the literature [23–27] related to LC–MS since this technique is currently one of the main approaches used for the quantitative analysis of small molecules in biological samples. However, the literature addressing this issue when CE–MS is applied is still scarce.

Several strategies have been suggested to minimize the interferences of matrix components that are not fully resolved such as the use of effective sample pre-treatment steps to reduce the presence of interfering compounds in the final extract or the use of an another ionization source. Alternatively, an appropriate calibration technique must be used to compensate for the signal alterations. The standard additions method represents the most-effective way to compensate for the adverse influence of the matrix [28]. Calibration with external matrix-matched standards is often proposed if analyte-free matrices are available. Another approach to compensate for matrix effects is based on the use of an adequate internal standard (structural analogue or isotopically labeled internal standard ILIS) [29]. It is generally believed that the use of an ILIS corrects for any matrix effects, but data reported in literature indicate that this issue needs proper attention [30,31].

Here we evaluated synthetic urine as a surrogate matrix for the accurate quantitation of these compounds by CE–ESI–MS, since no analyte-free samples of the authentic matrix are available. The standard additions method was used to evaluate the urinary levels of these naturally occurring compounds in healthy volunteers. Moreover, a comparison between the results obtained with CE–ESI–MS and another different method using LC–MS/MS in hydrophilic interaction chromatography mode coupled to a triple quadrupole detector is described. The results demonstrated that the new electrophoretic methodology constitutes a valid and reliable alternative for the accurate quantitative determination of the urinary levels of modified nucleosides in healthy individuals.

2. Material and methods

2.1. Chemicals

The analytical standards corresponding to *guanine* (Gua), CAS RN [73-40-5]; *7-methyl-guanine* (7mGua), CAS RN [578-76-7]; *1-methyl-guanine* (1mGua), CAS RN [938-85-2]; *9-methyl-guanine* (9mGua), CAS RN [5502-78-3]; *1-methyl-adenosine* (1mA), CAS RN [15763-06-1]; *cytidine* (Cyt), CAS RN [65-46-3]; *guanosine* (G), CAS RN [118-00-3]; *adenosine* (A), CAS RN [58-61-7]; *7-methyl-guanosine* (7mG), CAS RN [20244-83-4] were obtained from Sigma-Aldrich (Steinheim, Germany). Isotopically Labeled Internal Standards (ILISs), *8-^{13}C-guanine* (^{13}C-Gua) 98% and *ribose-2-^{13}C-adenosine* (^{13}C-A) 99%, were purchased from Cambridge Isotope Laboratories, Inc. (Massachusetts, USA).

Formic acid (>98%) and acetic acid (>98%) were from Fluka (Steinheim, Germany). Acetonitrile (ACN) and Methanol (MeOH) were of HPLC grade (Merck, Darmstadt, Germany). Ammonium formate (>98%) was from Riedel-de Häen (Hannover, Germany). Ammonia (solution at 25%) and 2-propanol were obtained from Scharlau (Barcelona, Spain). Ultra-high quality (UHQ) water was obtained with a Wasserlab water purification system (Noáin, Spain). All other chemicals were of analytical grade.

2.2. Instrumentation

All experiments were carried out with a Hewlett-Packard HP^{3D} CE instrument (Agilent Technologies, Waldbron, Germany) equipped with a UV–visible DAD device working at 260 nm with a bandwidth of 16 nm. MS was performed using an Agilent LC/MSD SL mass spectrometer (Agilent Technologies) equipped with a single quadrupole analyzer. Both the UV–visible DAD detector and the MS device were controlled by Agilent HP ChemStation software, version B.02.01 SR1.

Fused-silica capillaries (75 μm i.d., 375 μm o.d.) from Polymicro Technologies (Phoenix, AZ, USA), supplied by Composite Metal Services Ltd (West Yorkshire, UK), were used throughout the work. For CE–MS analysis, fused-silica capillaries (75 μm ID) with a total length of 87.5 and 20 cm to the UV detector were used; for CE-DAD analysis, the total capillary length was 58.5 cm and the effective length was 50 cm.

CE separation buffers and injection samples were placed in 1700-μL disposable amber glass vials with pre-perforated polyurethane caps (Agilent, Waldbroon, Germany).

2.3. CZE separation conditions

The running buffer (background electrolyte, BGE) was an aqueous solution of 100 mM formic acid–ammonium formate (pH 2.5). Injections were made at the anodic end, using a pressure of 50 mbar for 8 s. Electrophoretic separation was achieved with a voltage of 25 kV (normal polarity mode, detector at the cathodic end), with an initial ramp of 7 s. The temperature of the capillary was kept constant at 25 °C. Before its first use, the capillary was conditioned by flushing with the BGE for 10 min at 4 bar. This was also implemented as a daily start-up procedure. After each run, the capillary was rinsed at 4 bar for 1 min with fresh running buffer.

2.4. ESI–MS conditions

MS was performed using an Agilent LC/MSD SL mass spectrometer equipped with a single quadrupole analyzer. An Agilent coaxial sheath-liquid sprayer was used for CZE–ESI–MS coupling (Agilent Technologies). The sheath liquid consisted of 1:1 (v/v) isopropanol:UHQ water (7.5 mM acetic acid) and was delivered at a flow rate of 0.78 μL/min by an Agilent 1100 series pump, equipped with a 1:100 flow-splitter. The electrospray ESI voltage was set at +3500 V. Other ESI parameters under optimum conditions were: nebulizing gas pressure, 10 psi; drying-gas flow rate, 7 L/min; drying gas temperature, 150 °C. The optimized fragmentor voltage was 100 V for all analytes.

The mass spectrometer was operated in the positive-ion mode (ESI+). Analyte quantification was carried out under the selected ion monitoring (SIM) acquisition mode using protonated molecules $[M+H]^+$. A narrow isolation width of 1 *u* and dwell times of 52 ms were selected.

2.5. Preparation of standard solutions and quantitation

The initial stock standard solutions for the Gua (250 μg/mL), 7mGua, 1mGua, 9mGua, 1 mA, Cyt, G, A, 7mG (500 μg/mL) were prepared in acidified UHQ water (HCl 0.1 M). Two isotopically labeled internal standards (ILISs) were used: ribose-2-^{13}C-adenosine (^{13}C-A,) for free and modified nucleosides (A, 1mA, Cyt, G, 7mG), and 8-^{13}C-guanine (^{13}C-Gua) for the nucleobases (Gua, 1mGua, 7mGua, 9mGua); they were prepared in acidified UHQ water at 300 μg/mL. These stock solutions were stored at 4 °C in brown glass bottles.

Analyte quantification was carried out under SIM acquisition mode using protonated molecules, $[M+H]^+$. The quantitative variable used was the ratio between the peak area generated by the ion of each analyte, expressed in arbitrary units, and the peak area generated by the ILIS.

The values of the limits of quantification (LOQ), calculated from a signal to noise ratio of 10, between 0.10 μg/mL for Cyt, G and 9mGua and 0.36 μg/mL for 1mA were obtained.

2.6. Urine samples

Human urine samples from healthy volunteers (numbered from H1 to H5) were used to implement the method. Urine samples were collected in 250 mL brown glass bottles and frozen immediately until analysis. Before use, the urine samples were thawed at room temperature and filtered through 0.22 μm filters. A 1500-μL aliquot of natural urine was collected, adding 25 μL of a standard solution (300 μg/mL) of ^{13}C-Gua and ^{13}C-A, and then injected in the CE–MS instrument without any further treatment.

The urinary levels of the nucleosides were related to the urinary creatinine concentration which is a standard way to express the urinary levels of endogenous metabolites. This is justified because the excretion of creatinine is fairly constant over a longer time interval and allows randomly collected urine to be used instead of 24 h urine samples [32].

All urinary analyte concentrations were normalized against creatinine and expressed as nmol analyte/μmol creatinine. Urinary creatinine levels [33] were determined by a modified Jaffè method, based on the reaction between creatinine and picric acid, using photometric detection. The data on creatinine concentrations in the urine samples from the healthy volunteers were 0.99, 0.51, 0.52, 1.09, 0.74 in mg/mL, respectively for H1 to H5.

Normalization of the concentration values with respect to the creatinine content of the urine samples was accomplished using the following expression:

$$C_{\mathrm{Norm}}\ (\mathrm{nmol/\mu mol_{creat.}}) = \frac{C_{\mathrm{without\ Norm}}(\mathrm{\mu g/mL})}{C_{\mathrm{creat.}}(\mathrm{mg/mL}) \times M_{W_{\mathrm{analyte}}}} \times M_{W_{\mathrm{creat.}}}$$

where C_{Norm} is the normalized concentration expressed in units of nmol/μmol of creatinine; $C_{\mathrm{without\ Norm}}$ μg/mL) is the non-normalized concentration obtained with the standard additions method, $M_w$creatinine and $M_w$analyte represent the molecular weight of creatinine and the analyte, respectively, and $C_{\mathrm{creat.}}$ (in mg/mL) is the creatinine concentration, characteristic of each individual ($M_{w\mathrm{creat}}$, 113.12 g/mol).

3. Results and discussion

3.1. Optimization of CZE separation in acid medium

Owing to the scarce bibliography referring to the electrophoretic separation of these analytes in capillary zone electrophoresis (CZE) the electrophoretic behavior of nucleosides and nucleobases in different separation media – acid and basic medium – was first explored with a view to examining the possibilities offered by CZE in the separation of the analytes studied. To accomplish this, we used a UV diode-array detector (DAD) coupled with the CZE system.

The target compounds were guanine, 1-methyl-guanine, 7-methyl-guanine, 9-methyl-guanine; adenosine, 1-methyl-adenosine, cytidine, guanosine, 7-methyl-guanosine. As internal standards two isotopically ^{13}C labeled compounds, ribose-2-^{13}C-adenosine and 8-^{13}C-guanine, were used. Their structures are shown in Fig. 1.

It was observed that by using acidic medium as running buffer (100 mM formic acid, pH 2.5) these analytes were susceptible to becoming protonated and hence to migrating electrophoretically, which allows their separation with CZE.

In order to find the most suitable conditions, we performed a detailed study of the variables affecting the electrophoretic separation -the voltage applied and the composition of the separation medium (the presence of organic solvents, the concentration of the

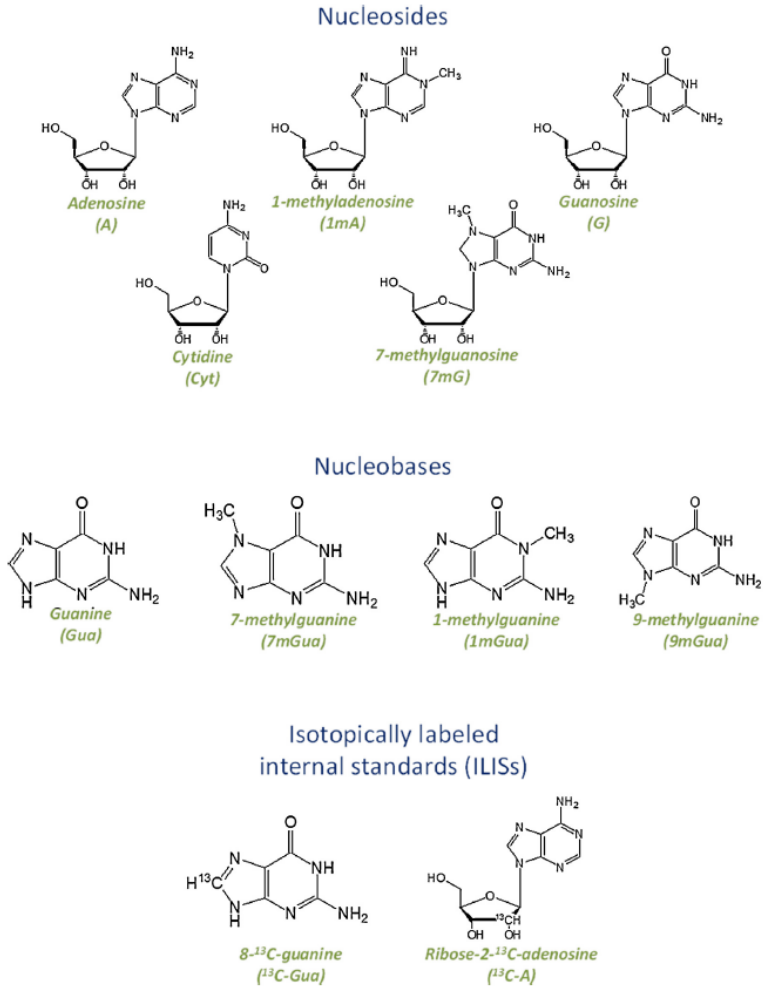

Fig. 1. Chemical structures of the compounds considered in this study.

background electrolyte)—and those affecting sample introduction (injection mode, medium and time of injection). For the optimization studies, we used an aqueous mixture of nucleosides and nucleobases at a concentration of 10 μg/mL.

First, electrophoretic separation was carried out by applying a constant voltage of 20 kV, which involves an excessively long time of analysis (>30 min). To reduce this we applied one voltage step (from 20 to 30 kV at 15 min), or two steps, from 20 to 25 kV at 12 min, and from 25 to 30 kV at 15 min. This latter action reduced the time of analysis to 22 min, such that it was selected for later use.

In order to transfer the CZE-UV DAD method to a CZE–MS application, special attention must be given to compatibility with MS requirements. For example, the concentration of the background electrolyte (BGE) used for electrophoretic separation should be optimized to keep the current intensity below 50 μA. The concentrations of the electrophoretic medium assayed were 50, 100, 150, 250 and 500 mM. According to the results on current intensity, it

was concluded that 100 mM was the optimum concentration for the background electrolyte (BGE).

To check to effect of an organic solvent in the separation medium we used methanol as a solvent at proportions between 2% and 30% (v/v). We decided to work in the absence of organic medium because in the presence of methanol no appreciable improvements in sensitivity were observed and, by contrast, an increase in the migration times was noted.

Sample injection into the capillary electrophoresis system can be performed using two injection modes: hydrodynamic and electrokinetic. The hydrodynamic injection mode was implemented by applying a positive pressure of 50 mbar over injection times ranging from 4 to 12 s. The results indicated that injection times between 5 and 8 s afforded suitable sensitivity and selectivity; these could not be improved with longer injection times. For the electrokinetic injection mode, injection voltages between 1 and 15 kV were studied, with different injection times: 5, 15 and 25 s. In all the experiments performed we obtained electropherograms with much less sensitive peaks than those obtained with hydrodynamic injection from samples of the same concentration. We also observed that the use of injection times longer than 5 s led to a noteworthy deformation of the electrophoretic peaks and a clear loss of resolution. In light of these results, we decided to use the hydrodynamic injection mode, applying a pressure of 50 mbar over 8 s.

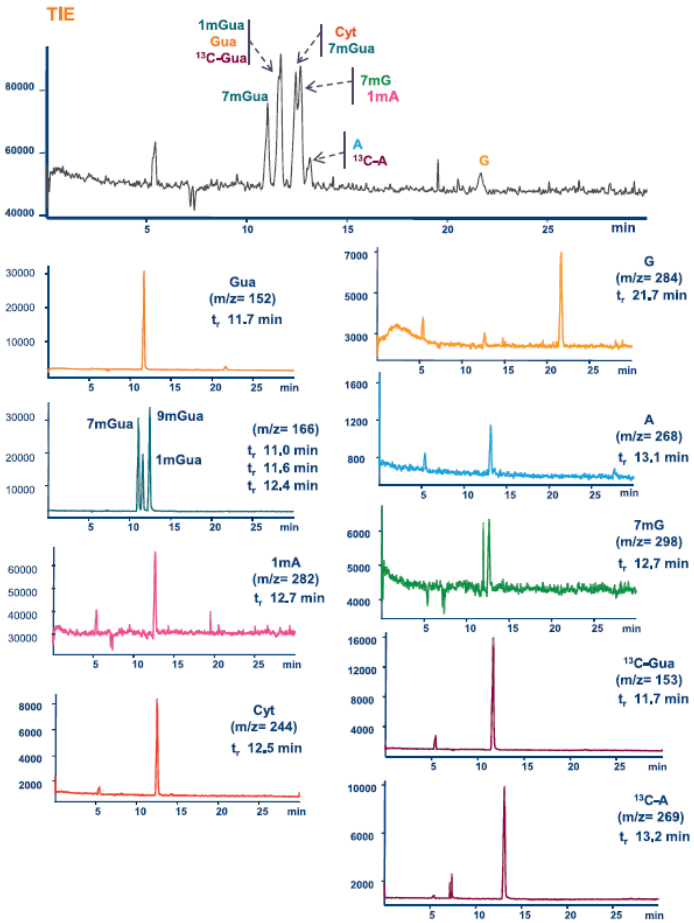

Fig. 2. Total ion electropherogram (TIE) and extracted ion electropherograms (XIE) for a mixture of nucleosides and nucleobases under optimal conditions. The figure includes the isotopically labeled compounds used as internal standards: ^{13}C-Gua and ^{13}C-A. The ions monitored were $[M+H]^+$ for all the analytes.

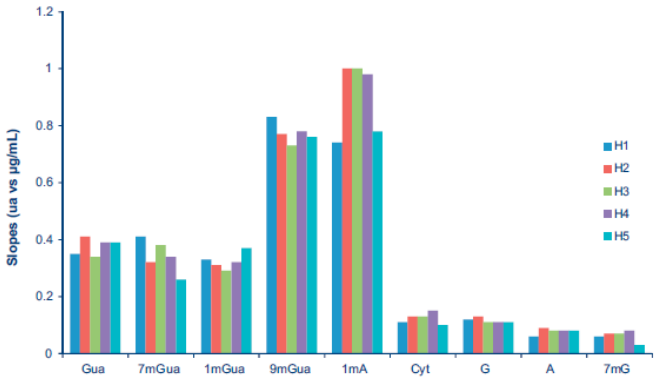

Fig. 3. Values of the slopes obtained by the standard additions method for each analyte in urine samples from healthy volunteers (H1–H5 urine samples).

3.2. CE–MS optimization

Here we used CE–MS with a coaxial electrospray interface (ESI) with sheath liquid, which together with the separation buffer closes the electric circuit. The nature and flow rate of the sheath liquid and the nebulizing gas pressure are decisive parameters in a CE–ESI–MS system since they govern the stability of the electric current, affect sensitivity since they determine the dilution of the sample, and favor the ionization process.

We studied the composition of the sheath liquid using water–ethanol and water–isopropanol mixtures at proportions of 1:1 and 1:2 (v/v) in the presence of 7.5 mM acetic acid or formic acid. The best results were obtained using a 1:1 (v/v) water–isopropanol mixture with 7.5 mM acetic acid.

We also optimized the cone voltage to study the possible fragmentation of the compounds analyzed; the cone voltage was varied between 40 and 150 V, and no fragmentation was observed for any of the analytes, but we observed greater sensitivity in the response when the cone potential applied was 100 V.

Fig. 2 shows the total ion electropherograms (TIE) and the extracted ion electropherograms (XIE) obtained by monitoring protonated molecular ions $[M+H]^+$ when working under the optimum conditions. It should be noted that the electrophoretic method developed here allowed the separation and differentiation between the 1mGua, 7mGua and 9mGua isomers, which possess identical molecular weights.

3.3. Application of the method to urine analysis

The difficulty involved in analyzing the endogenous metabolites studied here is related to the following aspects: (i) the different concentration levels at which these analytes are naturally present in the urine of the same individual, (ii) the marked variability shown by each analyte between different individuals and (iii) the lack of analyte-free samples of the authentic matrix.

In order to examine the response of naturally present urinary nucleosides, we analyzed urine samples from healthy volunteers. The collection and storage of the samples were carried out following the procedure reported in the experimental part.

3.4. Quantification by the standard additions method

To quantify these analytes it must be borne in mind that they are endogenous compounds and are naturally present in urine. Accordingly, it is not possible to find a natural matrix for use as a blank-matrix to prepare the calibration standards. In light of this, the standard additions method was used to evaluate the natural presence of these analytes.

The standard additions method is used when there is a possibility of a matrix effect occurring: that is, when an increase or decrease occurs in the signal due to the presence of other components. This effect is particularly important in the case of MS detection with electrospray ionization (ESI) since it has been demonstrated that this source of ionization is subject to matrix-dependent signal suppression or enhancement due to the presence of other compounds that coelute with the analytes of interest.

Quantification by standard addition was performed by spiking the urine samples, previously thawed and filtered, at three concentration levels: 0.5, 1.0 and 2.0 µg/mL. Additionally, the isotopically labeled internal standards (ILIs) were added to each aliquot at a concentration of 5 µg/mL. Thus, four aliquots per sample were obtained (the unspiked sample and three spiked aliquots) which were analyzed by CE–MS following the procedure described in Section 2.6.

Fig. 3 shows the slopes obtained with the standard additions method for each analyte in each of the five urine samples from the healthy volunteers; these slopes were obtained by linear regression of the straight line for the three additions made. Fig. 4 shows the electropherograms obtained for an unspiked urine sample directly injected into the CE–MS system. The naturally low level of 9mGua in healthy people can be appreciated (Fig. 4) while the signals for G and 7mG are hardly distinguishable.

The individual concentration values (in µg/mL) obtained with the standard additions method in the urine samples from the five healthy volunteers (H1–H5) are shown in Fig. 5. Note should taken of the great variability in the urinary level of the analytes of each sample from the same individual together with the variability between individuals. Thus, compounds such as 7mGua, 1mA and A were found at high concentrations (1–6.4 µg/mL), whereas Gua, 1mGua, 9mGua, Cyt, G and 7mG were at low-intermediate levels (<1 µg/mL). For some of these

Fig. 4. Total ion electropherogram (TIE) and extracted ion electropherograms (XIE) for a urine sample directly injected in the CE–MS system. The figure includes the isotopically labeled compounds used as internal standards: ^{13}C-Gua and ^{13}C-A. The ions monitored were $[M+H]^+$ for all the analytes.

latter analytes, such as 9mGua, G and 7mG, it would be appropriate to develop a preconcentration procedure since their natural levels were not adequate for accurate quantification in the all five samples of untreated urine. This can also be seen in Fig. 4.

In order to compare the levels of these compounds with other values reported for healthy people, normalization was performed with respect to the creatinine content (Table 1) as indicated in the experimental part (Section 2.6). The normalized values, expressed in nmol/μmol creatinine (Table 2) are in agreement with the values reported in the literature [18] for some of the compounds analyzed in healthy people.

3.5. Comparison of the results obtained with CE–MS and LC-QqQ

The electrophoretic CE–MS method was validated by comparing the urinary concentrations found in healthy individual with the results obtained by liquid chromatography with tandem mass spectrometry LC–MS/MS [34]. In this case, chromatographic separation was applied after a pre-treatment with a restricted-access material (RAM) of the urine sample prior to the injection in the LC–MS/MS system. However, for electrophoretic analysis untreated urine samples were used.

The LC–MS/MS [34] system employed was based on a stationary phase of hydrophilic interactions of the "zwitterionic" type

Table 1
Concentration levels of urinary nucleosides for healthy volunteers (H1–H5 urine samples) normalized against creatinine.

Analyte	H1 (0.99)[a] (nmol/$\mu mol_{creatinine}$)	H2 (0.51)[a] (nmol/$\mu mol_{creatinine}$)	H3 (0.52)[a] (nmol/$\mu mol_{creatinine}$)	H4 (1.09)[a] (nmol/$\mu mol_{creatinine}$)	H5 (0.74)[a] (nmol/$\mu mol_{creatinine}$)
Gua	0.28 ± 0.09	0.44 ± 0.18	1.54 ± 0.14	0.14 ± 0.03	0.35 ± 0.04
7mGua	2.36 ± 0.23	3.61 ± 0.16	1.96 ± 0.05	2.16 ± 0.14	5.95 ± 0.63
1mGua	0.12 ± 0.02	0.26 ± 0.05	0.22 ± 0.04	0.11 ± 0.01	0.24 ± 0.06
9mGua	Nd[b]	Nd	Nd	Nd	0.03 ± 0.04
1mA	1.57 ± 0.33	0.94 ± 0.05	0.72 ± 0.06	1.25 ± 0.11	2.05 ± 0.25
Cyt	0.10 ± 0.04	0.15 ± 0.01	0.11 ± 0.03	0.04 ± 0.01	0.21 ± 0.08
G	Nd	Nd	0.06 ± 0.02	0.09 ± 0.01	0.19 ± 0.17
A	1.51 ± 0.27	0.52 ± 0.01	0.89 ± 0.06	0.80 ± 0.04	1.14 ± 0.15
7mG	0.11 ± 0.02	Nd	Nd	0.07 ± 0.01	0.18 ± 0.05

[a] In brackets, creatinine value for each urine sample (in mg/mL).
[b] Not detected.

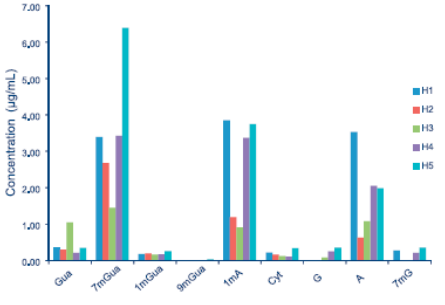

Fig. 5. Concentration levels (µg/mL) of urinary nucleosides and nucleobases from five healthy volunteers (H1–H5, unspiked urine samples) determined directly in the urine matrix by the standard additions method.

(ZIC-HILIC) coupled to a mass spectrometer with a triple quadrupole detector (QqQ) and an electrospray ionization source. This method was validated previously for the quantification of several nucleosides and nucleobases in urine according to the Guideline on Validation of Bioanalytical Methods [35].

Validation was performed for three human urine samples (H2, H4 and H5 samples) and for several of the modified nucleosides and nucleobases studied: 7mGua, 1mA, A. All experiments were carried out simultaneously with the CE–ESI–MS and LC–MS/MS setups. Fig. 6 shows the results obtained with both methods. These results were compared, applying a two-tailed *t* test in which a probability of $p > 0.05$ was obtained in all cases. Accordingly, it may be concluded that the results obtained with both methods were not significantly different.

Table 2
Comparison of levels of nucleosides excreted in urine reported in literature for healthy subjects with those obtained in the present work. Concentrations expressed in nmol/µmol creatinine.

Analyte	Literature data for healthy subjects[a]	Nucleoside levels for healthy subjects (this work)
A	0.18–4.70	0.52–1.51
Cyt	0.01–0.78	0.04–0.21
G	0.01–10.70	0.06–0.19
1mA	2.02–2.90	0.72–2.05

[a] Data from Ref. [18].

3.6. Quantitation using aqueous medium and synthetic urine as surrogate matrices

The standard additions method affords good results and, additionally, is an efficient approach to the common problem of matrix effects. However, it has the disadvantage of being laborious since the sample analyzed requires its own calibration plot after measuring the response for a series of standard additions. As an alternative, it was decided to assess the possibilities of quantification via the use of the external standard method.

Owing to the lack of analyte-free urine samples to prepare the calibration standards, for a reliable quantitation of endogenous compounds two approaches were attempted: calibration of an authentic analyte in a surrogate matrix and calibration of a surrogate analyte in the authentic matrix. Here we describe the first approach using pure water and synthetic urine as surrogate matrices for simultaneous quantitation of these endogenous compounds in human urine.

Calibration standards were prepared in pure water (UHQ) and in synthetic urine. The synthetic urine was prepared following the procedure described by van de Merbel [22], adding creatinine at a concentration of 1 g/L. In both cases, the calibration graphs revealed linear behavior within the range studied, 0.5–2 µg/mL.

Table 3 compares the mean value of slopes obtained in the authentic matrix by the standard additions method (H1–H5 urine samples, individual slopes shown in Fig. 3) with the slopes obtained by calibration in aqueous medium and by calibration in synthetic urine. For a good interpretation of the results, we applied a two-tailed *t* test to compare the mean slope values obtained in the authentic matrix by standard additions and the respective slopes obtained under the same experimental conditions but analyzing the standards in ultrapure water (UHQ) and in synthetic urine. On using UHQ water as the calibration matrix, the probability values (p) obtained were lower than 0.05 for some of the analytes studied, whereas in the case of calibration in synthetic urine it was observed that for all of the analytes the p value was greater than 0.05 indicating that there is no significant difference between the two slopes. Thus, surrogate matrix calibration in synthetic urine is proposed as an appropriate alternative to the method of standard additions.

These results can be explained bearing in mind that synthetic urine is prepared by trying to imitate the natural urine especially in regard to its salts content. Therefore the effect on the analytical signal obtained in the ESI–MS detector (signal suppression or enhancement) is expected to be similar to that undergone by the analytes in real urine.

Finally, in order to check the validity of using calibration in synthetic urine as an alternative to the standard additions method we compared the results obtained on analyzing a urine sample with both methods (Table 4) for the analytes showing intermediate or high concentration levels the results obtained with both

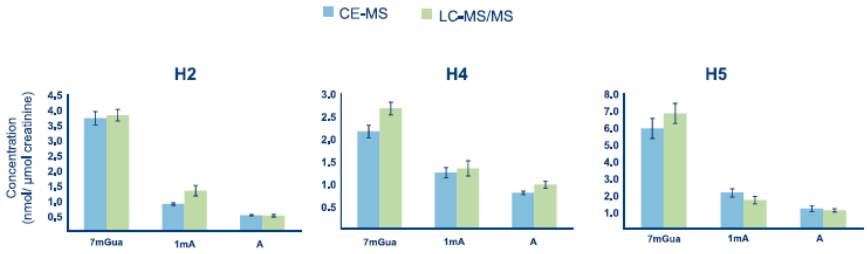

Fig. 6. Comparison of the results obtained by CE–MS and LC–MS/MS (nmol/µmol creatinine) for urine samples from healthy volunteers.

Table 3
Comparison of the slopes (expressed in arb. units vs. concentration in µg/mL) obtained in natural urine (H1–H5 samples) by the standard additions method and the slopes obtained by calibration in aqueous medium and in synthetic urine.

Analyte	Slope			$p_{SA\text{-}Synth}$ [b]	$p_{SA\text{-}Aq}$ [c]
	Standard addition method[a]	Calibration in synthetic urine	Calibration in aqueous medium		
Gua	0.38 ± 0.02	0.32 ± 0.03	0.34 ± 0.01	0.06	0.05
7mGua	0.34 ± 0.02	0.34 ± 0.02	0.40 ± 0.01	1.00	0.02
1mGua	0.32 ± 0.03	0.26 ± 0.03	0.33 ± 0.01	0.09	0.62
1mA	0.89 ± 0.09	0.65 ± 0.09	0.99 ± 0.03	0.05	0.17
Cyt	0.12 ± 0.02	0.13 ± 0.02	0.15 ± 0.01	0.58	0.10
A	0.08 ± 0.02	0.07 ± 0.01	0.06 ± 0.01	0.47	0.22

[a] Mean value of the five individual slope values obtained in urine samples H1 to H5 (shown in Fig. 2).
[b] Standard additions vs. Calibration in synthetic urine.
[c] Standard additions vs. Calibration in aqueous medium.

Table 4
Results for individual nucleosides and nucleobases in a sample of natural urine from a healthy volunteer analyzed with the standard additions method and via calibration in synthetic urine.

Analyte	Standard addition method (µg/mL)	Prediction by calibration in synthetic urine (µg/mL)	p
Gua	0.14 ± 0.02	0.10 ± 0.08	0.46
7mGua	1.83 ± 0.10	1.34 ± 0.27	0.06
1mGua	0.15 ± 0.05	0.11 ± 0.09	0.55
1mA	1.68 ± 0.18	1.57 ± 0.12	0.44
Cyt	0.10 ± 0.05	0.12 ± 0.09	0.76
A	0.88 ± 0.06	0.69 ± 0.10	0.07

methods did not differ significantly ($p > 0.05$). For those analytes present at low concentrations – 9mGua, G and 7mG – the need for a preconcentration step was confirmed since direct determination was difficult to perform. This enrichment step is now under development.

4. Concluding remarks

This paper describes the development of a rapid and robust methodology based on CZE–ESI–MS for the separation and determination of the urinary levels of a selected group of modified nucleosides and nucleobases (guanine, 1-methyl-guanine, 7-methyl-guanine, 9-methyl-guanine; adenosine, 1-methyl-adenosine, cytidine, guanosine and 7-methyl-guanosine).

The optimized method was applied to the separation of these endogenous compounds in human urine samples. Quantification of these endogenous compounds directly in the urine matrix was accomplished by applying the method of standard additions in order to evaluate the natural presence of these compounds in healthy volunteers. The results show the different urinary levels of these compounds for the same individual together with the variability between individuals. A comparison between the urinary levels of the compounds analyzed by CZE–ESI–MS and LC–MS/MS was performed, revealing that the new electrophoretic methodology constitutes a valid and reliable alternative to other existing methods such as liquid chromatography.

Here we evaluated the use of synthetic urine as a surrogate matrix for the accurate quantitation of these compounds as compared with the standard additions method. The good agreement in slopes suggests that both methods were comparable. Thus, the use of synthetic urine as a surrogate matrix for calibration is proposed as an appropriate alternative to the standard additions method for accurate quantification of these endogenous compounds by CZE–ESI–MS directly in the untreated urine from healthy people.

Acknowledgments

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Development of a procedure for the isolation and enrichment of modified nucleosides and nucleobases from urine prior to their determination by capillary electrophoresis–mass spectrometry

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ABSTRACT

A sample treatment step based on solid-phase extraction (SPE) with polymeric sorbents has been developed for the simultaneous isolation and preconcentration of nucleosides and nucleobases from urine prior to analyses by CE-ESI–MS. In most reported methods nucleosides are isolated from urine by SPE in affinity mode, using an immobilized phenylboronic acid group, which specifically binds *cis*-diols. However, this is not applicable to non-*cis*-diol compounds. Here, different types of polymeric sorbents were evaluated for the simultaneous extraction of nucleosides and nucleobases from urine. The best results were obtained with Isolute ENV+, a hydroxylated styrene–divylbenzene polymer, whose retention capacity can be attributed mainly to hydrophobic interactions, and thus it can be applied to a broad range of compounds, regardless of whether they present or not to the *cis*-diol group in their structure. Other parameters such as the elution solvent and sample volume were optimized. We also studied the influence of the addition of isotopically labeled internal standards (ILISs) before or after the extraction step. The detection limits achieved were in the 0.04–0.17 μg/mL range for a sample size of 2.0 mL and relative standard deviations were 4–22%. The whole method developed, SPE prior to CE-ESI–MS, was applied to human urine samples from healthy volunteers. We conclude that SPE with polymeric sorbents prior to the electrophoretic CE-ESI–MS methodology constitutes a fast, valid and reliable approach for the simultaneously extraction of urinary nucleosides and nucleobases.

1. Introduction

Modified nucleosides excreted in human urine are related to RNA turnover, a process that is increased in the presence of a tumor and other metabolic disorders. These nucleosides have been studied to examine their biomedical significance as possible biomarkers for cancer and other diseases.

Although these biomarkers lack sufficient specificity for use as single diagnostic tests, their clinical interest lies in their potential usefulness as previous complementary diagnostic methods and also to check the efficiency of treatment in patients undergoing treatment for cancer and in the follow-up of post-operative relapses. If a marker or group of markers is available for a given type of cancer, it is much easier to check the efficacy of the treatment by following the evolution of these compounds instead of repeating far more costly tests such as computerized tomography, and gammagraphy. Hence the importance of having available rapid and reliable analytical methods that can be used as routine tools for the determination of urinary levels of nucleosides in clinical laboratories.

During the last decade, many analytical methods for measuring and monitoring nucleosides in biological fluids have been reported, liquid chromatography with spectrophotometric [1–9] or MS detection [10–14] being the analytical technique most widely employed. Application of capillary electrophoresis for the determination of urinary nucleosides has also been reported; micellar electrokinetic capillary chromatography (MEKC), which involves the use of surfactants, is the mode usually applied [15–21]. However, this hinders MS detection. The application of other CE modes is still scarce.

Capillary zone electrophoresis coupled with electrospray ionization–mass spectrometry (CE-ESI–MS) has been previously assayed [22] for the determination of modified nucleosides in untreated human urine from healthy people. It has proved to be an appropriate approach for these compounds due to their hydrophilic character and also to the attractive features of CE–MS, since it combines the high separation capacity of CE with the good selectivity and high-power identification of mass spectrometry. Nevertheless,

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CE–MS currently presents a limited concentration sensitivity that may prevent its use in the analysis of compounds at trace levels. This is why the development and application of preconcentration techniques prior to CE–MS may be of special importance in fields were the quantification of compounds in a broad range of concentration levels is required. The need for efficient sample preparation is often a critical point in the total analysis. It may involve analyte isolation, trace enrichment and further cleanup to remove matrix interferents. These steps often take up most of the total analysis time, contributing strongly to the total cost of analysis and greatly influencing the reliability and accuracy of the analysis.

In literature, the techniques most frequently employed for the extraction of modified nucleosides from urine have been based on the use of an affinity interaction sorbent Affi-gel 601 [23–27]; this is a polymeric sorbent composed of polyacrylamide with immobilized groups of phenylboronic acid, which binds selectively to the *cis*-diol groups of the glucoside group. These bonds are only formed in nucleosides that have *cis*-diol groups at positions C-2 and C-3 of the ribose. This material does not retain nucleosides that do not have this *cis*-diol group and nucleobases such that they cannot be extracted from urine and it is necessary to search for an alternative for their extraction. It should be noted that in our revision of the literature we found abundant references to procedures using Affi-gel 601 in the extraction process of urinary nucleosides. However, few alternatives to the use of Affi-gel 601 in the extraction step have been reported [28,29].

The main aim of the present work was to develop an extraction and preconcentration procedure based on solid-phase extraction using polymeric sorbents which facilitates the simultaneous retention of nucleosides and nucleobases naturally present in urine, regardless of whether they have *cis*-diol groups or not. The target compounds were, guanine, 1-methylguanine, 7-methylguanine, 9-methylguanine; 7-methylguanosine, cytidine, adenosine and 1-methyladenosine. After the sample treatment procedure, the target analytes were separated and analyzed by capillary zone electrophoresis coupled to mass spectrometry (CZE-ESI–MS). The application to human urine from healthy volunteers is also reported.

2. Materials and methods

2.1. Chemicals

The analytical standard of *guanine* (Gua), CAS RN [73-40-5]; *7-methylguanine* (7mGua), CAS RN [578-76-7]; *1-methylguanine* (1mGua), CAS RN [938-85-2]; *9-methylguanine* (9mGua), CAS RN [5502-78-3]; *1-methyladenosine* (1mA), CAS RN [15763-06-1]; *cytidine* (Cyt), CAS RN [65-46-3]; *adenosine* (A), CAS RN [58-61-7]; *7-methylguanosine* (7mG), CAS RN [20244-83-4] were obtained from Sigma-Aldrich (Steinheim, Germany). Isotopically Labeled Internal Standards (ILISs), *8-^{13}C-guanine* (^{13}C-Gua), 98% and *ribose-2-^{13}C-adenosine* (^{13}C-A) 99% were purchased from Cambridge Isotope Laboratories, Inc. (Tewksbury, MA, USA).

Formic acid (>98%), acetic acid (>98%) were from Fluka (Steinheim, Germany). Acetonitrile (ACN) and methanol (MeOH) were of HPLC grade (Merck, Darmstadt, Germany). Ammonium formate (>98%) was from Riedel of Häen (Hannover, Germany). Ammonia (solution at 25%) and 2-Propanol were obtained from Scharlau (Barcelona, Spain). Ultra-high quality (UHQ) water was obtained with a Wasserlab (Noáin, Spain) Ultramatic water purification system. All other chemicals were of analytical grade.

2.2. Instrumentation

All experiments were carried out with a Hewlett-Packard HP^{3D} CE instrument (Agilent Tecnologies, Waldbron, Germany) equipped with a UV–visible DAD device. MS was performed using an Agilent LC/MSD SL mass spectrometer (Agilent Technologies) equipped with a single quadrupole analyzer. Both the UV–visible DAD detector and the MS device were controlled by Agilent HP ChemStation software, version B.04.01 SR1.

Fused-silica capillaries (75 μm ID, with a total length of 87.5 cm) were from Polymicro Technologies (Phoenix, AZ, USA), supplied by Composite Metal Scientific (Shipley, West Yorkshire, UK) and were used throughout the work.

CE separation buffers and injection samples were placed in 1700-μL disposable amber glass vials with pre-perforated polyurethane caps (Agilent, Waldbroon, Germany).

For SPE analysis, different types of sorbent (Oasis® HLB, Oasis® MCX, LiChrolut® EN, Isolute® ENV+) were assayed. Drying and conditioning of the cartridges were accomplished with a vacuum pump (Afa, Barcelona, Spain) coupled to a 20-port manifold for sample preparation (Varian, Harbor City, CA, USA).

2.3. Conditions for CE separation and ESI-MS detection

The running buffer (background electrolyte, BGE) was an aqueous solution of 100 mM formic acid–ammonium formate (pH 2.5). Injections were made at the anodic end, using a pressure of 50 mbar for 8 s. Electrophoretic separation was achieved with a voltage of 25 kV (normal mode), with an initial ramp of 7 s. The temperature of the capillary was kept constant at 25 °C. Before the first use, the capillary was conditioned by flushing with the BGE for 10 min at 4 bar. This was also implemented as a daily start-up procedure. After each run, the capillary was washed at 4 bar for 1 min with fresh running buffer.

MS was performed using an Agilent LC/MSD SL mass spectrometer equipped with a single quadrupole analyzer. An Agilent coaxial sheath-liquid sprayer was used for CE–MS coupling (Agilent Technologies). The sheath liquid consisted of 1:1 (v/v) isopropanol:UHQ water (7.5 mM acetic acid) and was delivered at a flow rate of 0.78 μL/min by an Agilent 1100 series pump, equipped with a 1:100 flow-splitter. The mass spectrometer was operated in the positive-ion mode (ESI+), with the ESI voltage set at +3500 V. Other electrospray parameters under optimum conditions were: nebulizer pressure, 10 psi; drying-gas flow rate, 6 L/min; drying gas temperature, 150 °C. The optimized fragmentor voltage was 100 V for all analytes.

2.4. Preparation of standard solutions and quantitation

The initial stock standard solutions for the Gua (250 μg/mL), 7mGua, 1mGua, 9mGua, 1mA, Cyt, A, 7mG (500 μg/mL) were prepared in acidified UHQ water (HCl 0.1 M). These stock solutions were stored at 4 °C in amber glass bottles. Two isotopically labeled internal standards (ILISs) were used: 8-^{13}C-guanine (^{13}C-Gua) for the nucleobases, and ribose-2-^{13}C-adenosine (^{13}C-A) for the nucleosides. They were prepared at 300 μg/mL and stored under the same conditions.

Analyte quantification was carried out under SIM acquisition mode using protonated molecules $[M+H]^+$. The quantitative variable used was the ratio between the peak area generated by the ion of each analyte, expressed in arbitrary units, and the peak area generated by the isotopically labeled internal standard (ILIS). Quantification was carried out by calibration in synthetic urine prepared according to Ref [30].

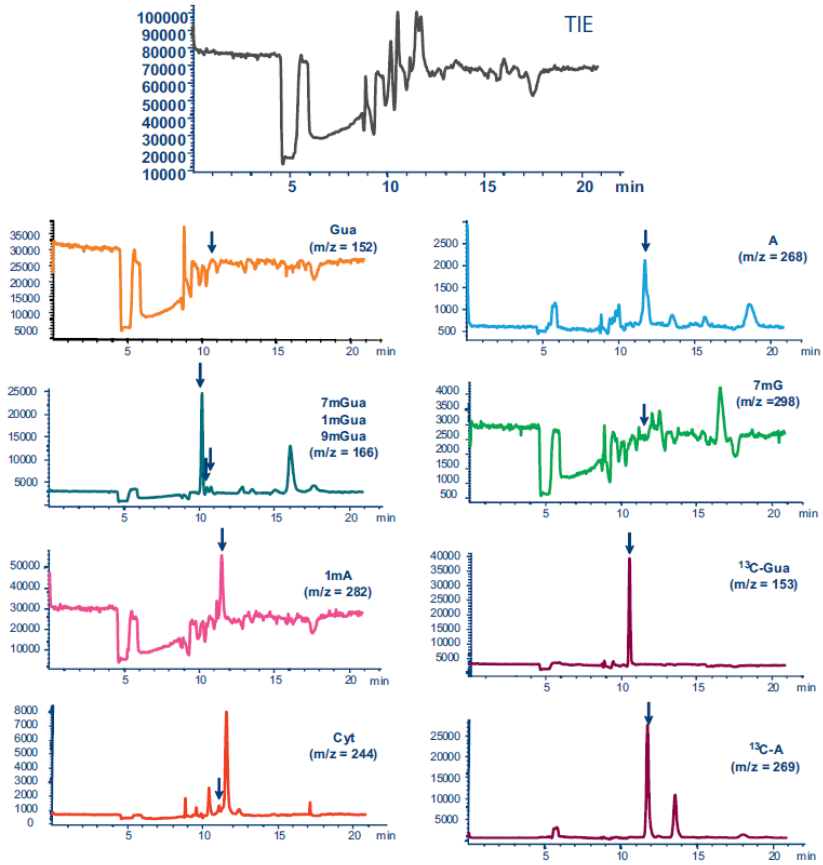

Fig. 1. Total ion electropherogram (TIE) and extracted ion electropherograms (XIE) of a urine sample from a healthy volunteer directly injected into the CE–MS. The figure includes the isotopically labeled compounds used as internal standards (ILISs): ^{13}C-Gua and ^{13}C-A. The ions monitored were $[M+H]^+$ for all the analytes.

2.5. Human urine samples

Samples of human urine from healthy volunteers were collected in 250 mL amber glass bottles and frozen immediately until analysis. Before use, the samples were thawed at room temperature. All samples were filtered through 0.22 μm filters prior to analysis.

Extraction–preconcentration was accomplished by passing 2.0 mL of urine through the extraction cartridge (Isolute ENV+); this cartridge was previously conditioned with 2 mL of acetonitrile and 2 mL of water (UHQ). Following this, the cartridges were dried for 15 min under a vacuum of −15 mm Hg. The analytes retained were then eluted with 2 mL of acetonitrile/ammonia (90:10, v/v). The eluted fraction was evaporated to dryness and the dry residues were reconstructed in 500 μL of water with formic acid 0.1% (v/v).

3. Results and discussion

Capillary electrophoresis coupled with mass spectrometry (CE–MS) has been previously described [23] for the determination of modified nucleosides in untreated human urine. Application of the method to urine samples revealed the broad concentration range at which these analytes are present in urine for the same individual, also observing broad variability for the same analyte between different individuals. Direct determination via CE-ESI–MS was possible for some of these compounds at medium or high concentration levels (for example 7mGua, 1mA, and A). For analytes present at intermediate and low concentrations, an enrichment step is required (Fig. 1).

Here we report the development of a procedure for the extraction and preconcentration of analytes whose levels in urine are

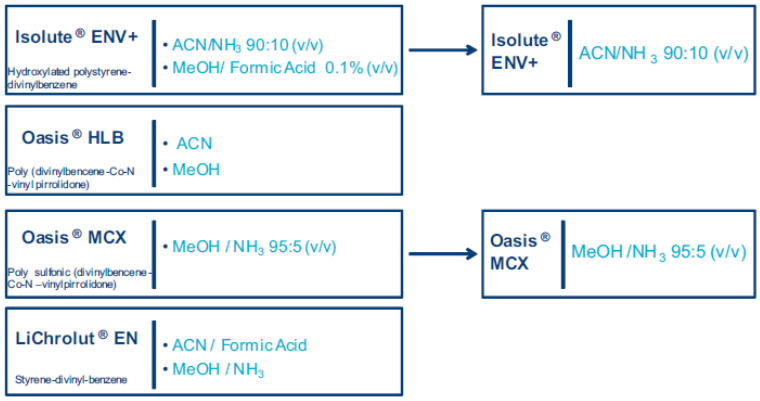

Fig. 2. Scheme of the optimization of the eluent/sorbent used for simultaneous extraction of nucleosides and nucleobases. Right side of the figure indicates the sorbent/eluent combination that afforded the best results for all compounds.

low and do not allow their direct determination via CE-ESI–MS. The target compounds were guanine, 1-methylguanine, 7-methylguanine, 9-methylguanine, adenosine, 1-methyladenosine, cytidine, 7-methylguanosine. Two isotopically labeled compounds, ribose-2-^{13}C-adenosine and 8-^{13}C-guanine, were used as internal standards. Electrophoretic separation was accomplished in CZE mode, using 100 mM formic acid at pH 2.5 as separation buffer. MS detection was accomplished in a single quadrupole, with the electrospray interface (ESI) operating in positive-ion mode.

3.1. Solid-phase extraction with polymeric sorbents prior to CE-ESI–MS

In order to facilitate the quantification of the modified nucleosides present in urine at low concentrations, we studied solid-phase extraction (SPE) with polymeric sorbents as an isolation and preconcentration method prior to separation by CE-ESI–MS.

We first assessed the ability of different sorbents to retain simultaneously the compounds of interest, nucleosides and nucleobases. The sorbents studied were: Isolute ENV+ (hydroxylated polystyrene–divinylbenzene), Oasis HLB (a copolymer of divinylbenzene-co-N-vinylpyrrolidone), Oasis MCX (a copolymer of sulfonic-divinylbenzene-co-N–vinylpyrrolidone), and LiChrolut EN (a copolymer of styrene divinylbenzene).

Different elution conditions were assayed depending on the nature of the sorbent. Fig. 2 shows the different sorbent/elution medium combinations tested. Volumes in the range 1–10 mL were assayed to elute the analytes from the cartridges. In all cases, an aqueous sample (1 mL) spiked with the analytes at a concentration of 1 μg/mL was used. Adjustment of the sample pH may be necessary to improve the retention of certain polar ionizable analytes that at the normal values found in urine are ionized and hence may be weakly retained by the sorbent.

In the case of the Oasis HLB, LiChrolut EN and ENV+ sorbents, due to their hydrophobic nature, pH control of the sample is not necessary because in the usual pH range of the urine from a healthy individual (between 4.6 and 8) the nucleosides and nucleotides are not ionized, which favors their retention. For Oasis MCX cartridges, a cation-exchange sorbent, retention studies were carried out in acidic media [29].

The best results were found for the ENV+ and Oasis MCX sorbents; the optimum eluent proved to be a mixture of acetonitrile/ammonia (90:10, v/v) for ENV+, and a mixture of methanol/ammonia (95:5, v/v) for the Oasis MCX. For the other two sorbents assayed, Oasis HLB and LiChrolut EN, low recoveries were obtained, such that no further studies were carried out with these sorbents.

The recovery values for the four sorbents studied, the mean of four replicas, are shown in Fig. 3. In this figure is also possible to note that the values of the standard deviations obtained for some analytes are relatively high. Accordingly, and to gain greater precision, in later studies we decided to work using the two ILISs described.

3.1.1. Influence of the sample volume

In light of the above results, ENV+ and MCX were chosen as suitable sorbents. In order to achieve higher enrichment factor, and thereby improve the sensitivity of the method, a study was made of the effect of the volume of sample to be preconcentrated. The improvement in detection limits based on an increase in the volume of sample has some limitations, such as the fact that retention capacity of the sorbent might be surpassed or that, for highly polar analytes, the breakthrough phenomenon may occur. There is also a practical limitation, i.e. the time involved in the passage of large sample volumes.

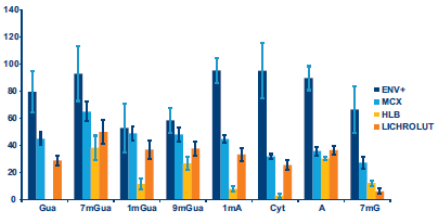

Fig. 3. Recoveries ($n = 4$) obtained after solid-phase extraction with different polymeric sorbents, ENV+, Oasis MCX, Oasis HLB and LiChrolut EN.

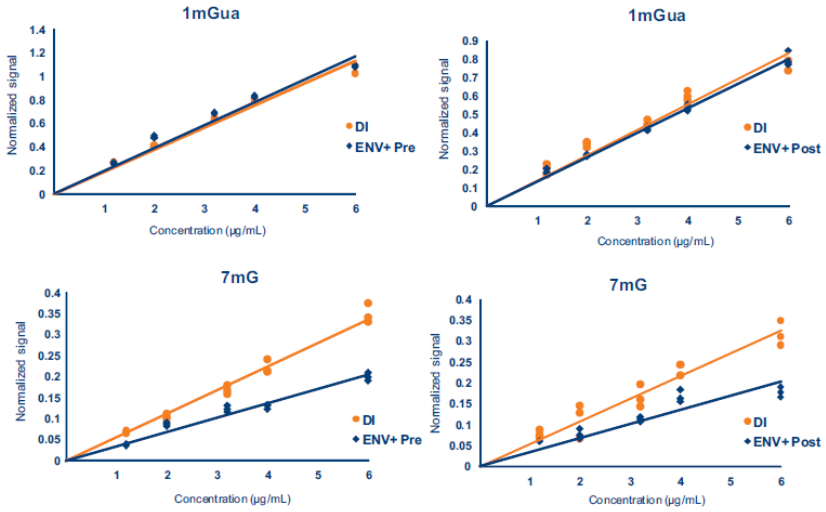

Fig. 4. Calibration lines obtained by addition of ILISs before or after the SPE step. Comparison with the calibration lines obtained by direct injection (DI) of synthetic urine standards.

Table 1
Calibration lines obtained by addition of internal standards (ILISs) before or after the SPE step. Comparison with the calibration line in synthetic urine obtained by direct injection (DI) without preconcentration. Recovery (%) = (m_{ENV+}/m_{DI}) × 100 calculated as the ratio between the slope of the regression line obtained with preconcentration (m_{ENV+}) and the one generated by direct injection (m_{DI}).

Analytes	Slope			Recovery %	
	m_{DI} [a]	$m_{ENV+Pre}$ [b]	$m_{ENV+Post}$ [c]	ILIS before SPE ($m_{ENV+Pre}/m_{DI}$)	ILIS after SPE ($m_{ENV+Post}/m_{DI}$)
Gua	1.75	1.61	1.42	92	81
7mGua	1.17	1.32	1.12	113	96
1mGua	0.59	0.65	0.53	110	90
9mGua	1.36	1.28	0.81	94	70
1mA	2.48	2.65	2.00	107	81
Cyt	0.66	0.55	0.60	83	91
A	0.31	0.28	0.31	90	100
7mG	0.22	0.12	0.13	65	68

[a] Calibration line in synthetic urine obtained by direct injection, without preconcentration.
[b] Calibration line in synthetic urine with preconcentration with ENV+ cartridges by adding the ILISs before the SPE step.
[c] Calibration line in synthetic urine with preconcentration with ENV+ cartridges by adding the ILISs after the SPE step.

The experiments were carried out with sample volumes ranging between 1 and 10 mL of natural urine. Because the compounds were endogenous, the study was performed with unspiked urine samples. Satisfactory results were obtained with both cartridges, showing that the analytical signal increased with increase in the sample volume loaded into the cartridge. Thus no breakthrough effects were observed. In later studies, it was decided to use the ENV+ sorbent and a volume of urine of 2 mL as a compromise between the increase in sensitivity and the time taken in the SPE step.

Table 2
Analytical characteristic of the method developed based on SPE with ENV+ prior to CE-ESI–MS.

		Gua	7mGua	1mGua	9mGua	1mA	Cyt	A	7mG
Calibration parameters	Intercept (10^{-2})	−3 ± 6	−3 ± 4	−1 ± 2	−3 ± 2	−3 ± 8	−5 ± 1	0 ± 1	1 ± 1
	Slope (AU·mL/µg)	1.44 ± 0.04	1.19 ± 0.04	0.53 ± 0.02	0.90 ± 0.02	2.00 ± 0.09	0.71 ± 0.02	0.30 ± 0.01	0.13 ± 0.01
	CC_α [a] (µg/mL)	0.06	0.07	0.07	0.05	0.09	0.04	0.08	0.17
Repeatability as RSD (%) [b]	0.3 µg/mL	11	14	12	18	5	12	7	22
	1.5 µg/mL	9	14	4	12	9	17	7	17

[a] CCα: decision Limits, calculated according to the 2002/657/EC decision.
[b] RSD: relative standard deviation (n = 6).

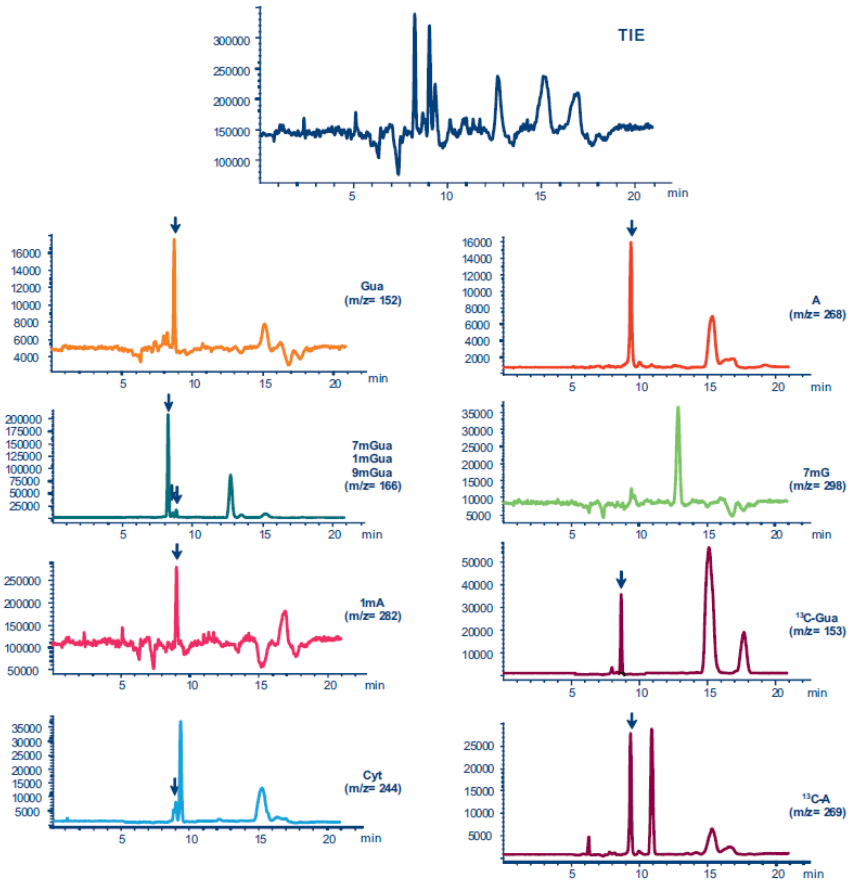

Fig. 5. Total ion electropherogram (TIE) and extracted ion electropherograms (XIE) of a urine sample from a healthy volunteer after SPE step with ENV+ prior to CE-ESI–MS.

3.1.2. Influence of the addition of isotopically labeled internal standards (ILISs) during the SPE step

One of the problems that arise when working with ESI-MS detection is the fluctuation in the signals due to ion suppression effects that occurs in the electrospray device. These effects are mainly due to the co-elution of matrix interferents with the analytes, such that it is necessary to use one or several internal standards for suitable quantification to be achieved. Here we worked with two isotopically labelled internal standards (ILISs): 8-^{13}C-guanine (^{13}C-Gua) and ribose-2-^{13}C-adenosine (^{13}C-A). The former was used as the internal standard of Gua, 7mGua, 9mGua and 1mGua; whereas ^{13}C-A was used as the internal standard of 1mA, Cyt, A and 7mG.

We then studied the influence of adding the ILISs before or after the solid-phase extraction step (SPE). In the first case (pre-SPE) the quantitative recovery of the overall process was assessed, while in the second one (post-SPE) the aim was to correct the variability in the signal obtained at the MS detector.

For this study, synthetic urine [31] was employed as a surrogate matrix because these compounds are naturally present in human urine. Aliquots of 2 mL of synthetic urine were spiked at five concentration levels, 0.3, 0.5, 0.8, 1.0 and 1.5 μg/mL, and passed through the ENV+ cartridge. After elution of the SPE sorbent, the solvent was evaporated off and the dry residue was redissolved in 500 μL of an aqueous solution of 0.1% (v/v) formic acid for further analysis by CE-ESI–MS. Thus, the preconcentration factor was 4. The internal standards, ^{13}C-Gua and ^{13}C-A, were added at two different concentrations: 1 μg/mL, when they were added before the preconcentration step (pre-SPE), and 4 μg/mL when they were added after that step (post-SPE). For each concentration level, two ENV+ cartridges were used and the final extract was injected in triplicate.

Table 3
Comparison of the limits of detection (LODs) for several methods reported in the literature (2007-2013) for the determination of modified nucleosides and nucleobases in urine.

Analytes	Sample treatment	Analytical technique	LOD (ng/mL)	Reference
5 Nucleosides	On-line solid-phase microextraction (SPME)	Capillary LC-UV (home-made HILIC column)	40–52	[9]
6 Nucleosides 2 Nucleobases	On-line solid-phase extraction (SPE) with RAM	LC–MS/MS (ZIC-HILIC)	0.1–1.3	[12]
8 Nucleosides	–	UHPLC-UV (Kinetex C_{18})	23–36	[13]
12 Nucleosides	Phenylboronic acid (PBA) column	LC–MS/MS (C18)	40–180	[14]
7 Nucleosides	Solid-phase extraction (SPE) with Oasis MCX	LC–MS/MS (C18)	7.5–75	[29]
11 Nucleosides	Affi-Gel 601	MEKC-UV	150–406	[21]
14 Nucleosides	Affi-Gel 601	MEKC-UV	40–580	[24]
11 Nucleosides	Affi-Gel 601	CE–MS	2.4–1024	[27]
5 Nucleosides 3 Nucleobases	Solid-phase extraction (SPE) with Isolute ENV+	CE–MS	40–170	This work

As a reference, a calibration line was obtained by direct injection of synthetic urine standards spiked at the level expected if recoveries were 100%; that is, 1.2, 2.0, 3.2, 4.0, and 6.0 μg/mL. It should be borne in mind that the preconcentration factor is 4 for the procedure described (the sample volume to be preconcentrated is 2.0 mL and the final volume is 0.5 mL. Fig. 4 shows the results for 1mGua and 7mG as examples of the more and less favorable behavior, respectively.

The slope of the regression line obtained with the SPE step (m_{ENV+}) and the one generated by direct injection (m_{DI}) of standards in synthetic urine were calculated. The ratio between the slopes of both lines, that is, m_{ENV+}/m_{DI}, represents the mean recovery along the whole concentration range studied. Table 1 shows the recoveries R (%), expressed as ($m_{ENV+Pre}/m_{DI}$) and ($m_{ENV+Post}/m_{DI}$) for the addition of ILISs before and after the SPE step, respectively.

In the case of experiments involving the addition of the ILISs before the preconcentration step (pre-SPE) the values of $m_{ENV+Pre}/m_{DI}$, expressed in %, were in the 65–113% range (Table 1). Upon adding the ILISs after the preconcentration step (post-SPE) the $m_{ENV+Post}/m_{DI}$ values ranged between 68% and 100%. Accordingly, it may be seen that the addition of the ILISs before or after the preconcentration step does not afford significantly different results. This may be due to the use of isotopically labeled compounds as internal standards, which showed a similar behaviour on the SPE step as the analytes of interest.

3.1.3. Analytical characteristics of the method based on SPE prior to the CE-ESI–MS

The analytical characteristics of the CE-ESI–MS method after SPE with ENV+ sorbents were determined (Table 2) taking into account all the above-described conditions. Calibration graphs were obtaining by using a sample volume of 2 mL and the ILISs were added after the SPE step. The results reveal a good linear relationship between the analytical signals normalized with the internal standard against the analyte concentration in the 0.2–5 μg/mL range.

The precision of the method was determined from the values of the relative standard deviation in percentage form (%), calculated for two concentration levels of 0.3 and 1.5 μg/mL. It is seen that for both levels the values of the coefficient of variation range between 4 and 22%. These values can be considered acceptable, bearing in mind that the procedure involves a preconcentration step that requires a change of the solvent due to evaporation of the extract eluted from the cartridge and later redissolution of the dry extract in a medium suitable for electrophoretic separation.

The decision limits, CCα, were calculated from the calibration curves following the criterion proposed in the 657/2002/CE Decision. The values found ranged between 0.04 and 0.17 μg/mL for Cyt and 7mG respectively. Table 3 shows a comparison of the limits of detection (LODs) obtained with the different methods reported in the literature in recent years. It can be seen that the method proposed here affords better LODs than others methods based on CE-UV; in comparison with LC–MS/MS using a SPE step with phenylboronic acid, similar results were obtained, but in our case the use of a polymeric sorbent allowed us to perform the simultaneous extraction of nucleosides and nucleobases.

3.1.4. Application to human urine samples

The proposed extraction–preconcentration procedure was applied to the simultaneous determination of modified nucleosides and nucleobases in urine from a healthy volunteer.

In view of the lack of certified samples of modified nucleosides in urine, the method was validated by determining the recovery at two concentration levels in urine from a healthy volunteer. To accomplish this, we analyzed an unspiked urine sample according to the SPE procedure prior to CE-ESI–MS. Fig. 5 shows the electropherograms obtained; when compared to Fig. 1 they show that the SPE step provides cleaner electropherograms. Moreover, in the non-preconcentrated sample it is not possible to quantify the analytes Gua, Cyt and 7mG, whereas in the sample obtained after the SPE step (Fig. 5) the first two can be quantified.

The sample was then spiked at two levels, 0.5 and 1 μg/mL. The signal obtained for each analyte was introduced into the corresponding calibration line; the quantitative variable used was the

Table 4
Results obtained for a sample of human urine analyzed by the proposed SPE step with ENV+ prior to CE-ESI–MS.

Analytes	Added (μg/mL)	Found (μg/mL)	Found by SA[a] (μg/mL)
Gua	0.0	0.08	0.09
	0.5	0.61	
	1.0	1.08	
7mGua	0.0	2.30	2.33
	0.5	2.83	
	1.0	3.47	
1mGua	0.0	0.11	0.12
	0.5	0.63	
	1.0	1.15	
9mGua	0.0	<LOD	–
	0.5	0.41	
	1.0	0.82	
1mA	0.0	1.09	1.15
	0.5	1.58	
	1.0	1.91	
Cyt	0.0	0.06	0.06
	0.5	0.55	
	1.0	1.11	
A	0.0	4.46	4.50
	0.5	5.14	
	1.0	5.50	
7mG	0.0	<LOD	–
	0.5	0.39	
	1.0	0.80	

[a] Found with the standard addition (SA) method directly in the unspiked urine sample.

ratio between the peak area generated by the ion of each analyte and the peak area generated by the ILIS. The ratio thus obtained was quantified according to the calibration line obtained by the direct injection of external standards. The amounts found (Table 4) for all the analytes studied matched the expected values satisfactorily, taking into account the additions made.

Accuracy was checked by analyzing unspiked urine sample by the standard additions method and comparing the results with those obtained with SPE prior to CE-ESI–MS. The results of the unspiked urine sample with the standard additions method are also shown in Table 4. It should be noted that in the case of the 9mGua and 7mG it was not possible to perform their quantitation in the unspiked sample, using the standard additions method owing to the low levels at which they were present.

4. Conclusions

In the present work we report the development of a procedure based on solid-phase extraction (SPE) for the isolation and preconcentration of urinary nucleosides and nucleobases naturally present in urine at low concentration levels, which do not allow their direct determination via CE-ESI–MS.

The results indicate that the polymeric sorbent (ENV+), a hydroxylated copolymer of polystyrene divinylbenzene, can be satisfactorily applied for the simultaneous retention of nucleosides and nucleobases regardless of the presence or absence of the *cis*-diol group in their structure. The proposed procedure used only 2 mL of urine but sample volume can be easily increased in order to maximize the sensibility. Recoveries in the range 65–113% were found for all the target compounds with detection limits in the 40—170 ng/L range.

The applicability of the proposed procedure in human urine from healthy people was tested. The endogenous levels of these urinary compounds were confirmed using the method of standard additions for quantification directly in the untreated urine matrix. This confirms that the methodology developed, SPE with a polymeric sorbent prior to CE-ESI–MS, is a suitable approach when wishing to perform the simultaneous determination of modified nucleosides and nucleobases in urine at low concentration levels.

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ORIGINAL ARTICLE

Adenosine monophosphate is elevated in the bronchoalveolar lavage fluid of mice with acute respiratory toxicity induced by nanoparticles with high surface hydrophobicity

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Abstract

Inhaled nanomaterials present a challenge to traditional methods and understanding of respiratory toxicology. In this study, a non-targeted metabolomics approach was used to investigate relationships between nanoparticle hydrophobicity, inflammatory outcomes and the metabolic fingerprint in bronchoalveolar fluid. Measures of acute lung toxicity were assessed following single-dose intratracheal administration of nanoparticles with varying surface hydrophobicity (i.e. pegylated lipid nanocapsules, polyvinyl acetate nanoparticles and polystyrene beads; listed in order of increasing hydrophobicity). Broncho-alveolar lavage (BAL) fluid was collected from mice exposed to nanoparticles at a surface area dose of 220 cm^2 and metabolite fingerprints were acquired via ultra pressure liquid chromatography-mass spectrometry-based metabolomics. Particles with high surface hydrophobicity were pro-inflammatory. Multivariate analysis of the resultant small molecule fingerprints revealed clear discrimination between the vehicle control and polystyrene beads ($p < 0.05$), as well as between nanoparticles of different surface hydrophobicity ($p < 0.0001$). Further investigation of the metabolic fingerprints revealed that adenosine monophosphate (AMP) concentration in BAL correlated with neutrophilia ($p < 0.01$), CXCL1 levels ($p < 0.05$) and nanoparticle surface hydrophobicity ($p < 0.001$). Our results suggest that extracellular AMP is an intermediary metabolite involved in adenine nucleotide-regulated neutrophilic inflammation as well as tissue damage, and could potentially be used to monitor nanoparticle-induced responses in the lung following pulmonary administration.

Keywords

AMP, broncho-alveolar lavage, hydrophobicity, inflammation, lung, metabolomics, nanoparticles

History

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Introduction

Safety assessment for inhaled nanomaterials is complex and advances in respiratory nanotoxicology depend upon developing a mechanistic understanding to link physical and chemical properties to measurable toxicological outcomes. According to some classifications, nanomaterials may be divided into high- and low-toxicity materials based on their dose-response in the lungs (Aitken et al., 2009; Fadeel et al., 2012). High-toxicity nanoparticles which induce inflammation at low doses are generally composed of highly reactive materials, e.g. materials with significant positive or negative surface charge or materials that become toxic upon intracellular processing (Cho et al., 2012). In contrast, low-toxicity nanomaterials (e.g. titanium dioxide, gold, silver and polystyrene) tend to present a much more inert surface, hence they require much higher exposure doses to induce pulmonary inflammation (Donaldson et al., 2008; Duffin et al., 2007). This response to low-toxicity nanoparticles is attributed primarily to the high material burden rather than nanomaterial reactivity (Maynard et al., 2011).

Most of the studies of low-toxicity nanomaterials have been conducted using rigid, crystalline, insoluble materials, whereas there has been less research on the response of the respiratory system to the administration of organic nanomaterials (Beyerle et al., 2011; Dailey et al., 2006; Harush-Frenkel et al., 2010; Liu et al., 2009; Nassimi et al., 2009), sometimes referred to as soft nanomaterials (Nalwa, 2009). Organic nanomaterials are increasingly being developed as inhaled nanomedicines or components of aerosol-based consumer products, e.g. hairsprays, cleaning products, and include nanoconstructs such as liquid crystals, proteins, nucleic acids, polymers, surfactants, micelles or emulsions (Nalwa, 2009). These have very different physico-chemical properties compared to rigid crystalline nanoparticles and typically present an amorphous, more flexible surface that may be highly hydrated (Dailey, 2009; Maynard et al., 2011;

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Moghimi & Szebeni, 2003; Lorusso et al., 2007). Hence, it is important to evaluate how the respiratory system responds to the physicochemistry presented by organic nanomaterials, especially when these are biopersistent.

Surface hydrophobicity is one feature of many organic nanoparticles and has been identified as a defining factor in how the lungs respond to their administration (Beyerle et al., 2011; Maynard et al., 2011). In this study, hydrophobic interaction chromatography (HIC) (Carstensen et al., 1991) was used to characterise five different nanoconstructs, designed to possess increasing surface hydrophobicity. The nanoconstructs included two different lipid nanocapsule (LNC) formulations, two types of polyvinyl acetate (PVAc) nanoparticles, and commercially available polystyrene beads as a control material to benchmark to the literature. Nanoparticles were administered intratracheally (i.t.) to mice. Bronchoalveolar lavage (BAL) fluid was collected at 24 h and evaluated for markers of inflammation and tissue damage. Neutrophil counts and cytokine levels in BAL confirmed that the nanoparticles induced an inflammatory response linked to increasing surface hydrophobicity, with responses similar to those reported for known low-toxicity nanomaterials (Brown et al., 2001; Dailey et al., 2006; Duffin et al., 2007).

In metabolomics research, it is conventional to develop investigations through a two phase approach. In the first phase, non-targeted metabolomics is used to screen for differences in metabolite levels that indicate perturbations in normal metabolic pathways. The strength of this approach is that it is non-biased towards any particular outcome (i.e. non-hypothesis driven) and may therefore identify putative markers of both known and previously undiscovered metabolic pathways. Once metabolites of interest have been identified, targeted phase two studies are designed to investigate specific pathways of interest, evaluate multiple compartments or perform longitudinal studies. The primary aim of this study was to apply non-targeted metabolomics to assess whether nanoparticle-induced acute respiratory toxicity yields informative metabolite profiles in BAL fluid and identify putative markers of nanoparticle-induced toxicity. It was anticipated that BAL fluid from vehicle control animals would differ significantly in metabolite profile compared to that of animals exposed to nanoparticles. Further, it was postulated that specific metabolites may be identified which correlate with the acute respiratory toxicity induced by nanoparticles of increasing surface hydrophobicity.

Methods

Polymer synthesis

Two grades of PVAc, high-molecular weight (148 kDa) and low-molecular weight (12.8 kDa), were purchased from Sigma Aldrich, Dorset, UK. The 148 kDa PVAc was subjected to direct saponification according to the method described by Chana et al. (2008) producing a modified PVAc polymer with 17 mol% hydroxyl groups and 83 mol% residual acetate groups (PVAc80). The 12.8 kDa PVAc polymer was subjected to direct saponification under different reaction conditions to produce a PVAc polymer with 34 mol% hydroxyl groups and 66% residual acetate groups (PVAc60) (Chana et al., 2008). Polymer purity and degree of hydrolysis were verified by NMR analysis prior to use. Poly vinyl alcohol (PVA; 8–12 kDa; 80 mol% hydroxyl; 20 mol% acetate groups) was used as a stabiliser in the manufacturing of nanoparticle and was purchased from Sigma Aldrich, Dorset, UK.

Manufacture of PVAc nanoparticles

PVAc60 nanoparticles were prepared according to a method by Chana et al. (2008) by injecting a solution of 5% w/v PVAc60 dissolved in 2:1 methanol:water into a 0.33% w/v aqueous solution of the stabiliser, PVA, whilst stirring at 3500 rpm. PVAc80 nanoparticles were prepared by injecting a solution of 1% w/v PVAc80 dissolved in 2:1 methanol:water into a 0.33 % w/v aqueous solution of the stabiliser, PVA, whilst stirring at 3500 rpm. Following 30 min constant stirring at 4000 rpm and solvent evaporation overnight (~100 rpm), the nanosuspensions were dialysed against water (72 h) to remove excess PVA and subsequently concentrated to the desired final concentration using ultrafiltration centrifuge tubes (Millipore Corporation, Billerica, MA; 100 kDa MWCO). Residual PVA was <0.4–0.5 mg/mL. Particles were stored at 4 °C and were used within one week of preparation.

Manufacture of LNCs

LNCs were manufactured using a phase-inversion temperature method (Heurtault et al., 2003). LNCs with a diameter of ~50 nm (LNC50) were prepared by generating a coarse emulsion of 17% w/v Labrafac® WL1349 (Gattefosse, Saint-Priest, France), 17.5% w/v Solutol® HS15 (BASF, Ludwigshafen, Germany), 1.75% w/v Lipoid® S75-3 (Lipoid GmbH, Ludwigshafen, Germany) and 3% w/w NaCl in purified water. This emulsion was then submitted to repeated heating cooling cycles (85°–60°–85°–60°–85 °C) before cooling to 72 °C at which point ice-cold water was added. Excess stabiliser (Solutol® HS15) was removed from the suspension by dialysis (72 h) against water containing BioBeads® (BioRad, Hertfordshire, UK) and subsequent concentration using ultrafiltration centrifuge tubes (Millipore Corporation, Billerica, MA; 100 kDa MWCO). Residual Solutol HS 15 content was determined to be <0.5 mg/mL. LNC with a diameter of ~150 nm (LNC150) were prepared and purified using the same method, except that the relative concentrations of the three main coarse emulsion components were: 25% w/v Labrafac WL1349, 8.5% w/v Solutol® HS15, 1.75% w/v Lipoid S75-3 (Lipoid GmbH, Ludwigshafen, Germany) and 3% w/w NaCl in purified water. Particles were stored at 4 °C and were used within one week of preparation.

Polystyrene nanoparticles

Unmodified polystyrene nanoparticles with a diameter of 50 nm (PS50; 2.62% m/v) were used as a control and were purchased from Polysciences (Eppelheim, Baden-Württemberg, Germany).

Nanoparticle size and surface charge

Particle size and zeta potential were determined using a Zetasizer Nano ZS (Malvern, Worcesterchire, UK). Particle suspensions were diluted in the test medium (purified water, 6.3 mM sodium chloride or Hank's buffered saline solution containing 10% fetal bovine serum) prior to measurement and the analysis parameters (viscosity, temperature, refractive index) were adjusted to match the medium and sample type used for analysis.

Nanoparticle hydrophobicity

Surface hydrophobicity of nanoparticle suspensions ($n = 3$ three individual batches) was assessed using HIC (Carstensen et al., 1991). Briefly, 250 µL nanoparticle suspension was prepared in phosphate-buffered saline (PBS) and eluted through three different HiTrap™ substituted sepharose hydrophobic interaction columns: Butyl FF, Phenyl FF (high substitution) and Octyl FF (GE Healthcare Life Sciences, Little Chalfont, UK). Fractions of 1 mL elutant were collected and analysed for particle content via turbidity measurement (Lambda 35; Perkin-Elmer, Cambridge, UK; $\lambda = 450$ nm). The column-bound particle fraction was subsequently eluted from the column using 0.1% Triton X-100 and turbidity measured. Absorbance values were plotted against

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elution volumes and two area under the curve (AUC) values were calculated using Origin™ software. The percentage particle retention in each of the three columns was defined as:

$$\%\ \text{Column retention}\ (\%\text{R}) = \left(\frac{\text{AUC } \textit{TritonX}}{\text{AUC } \textit{PBS} + \text{AUC } \textit{TritonX}}\right) \times 100 \quad (1)$$

The HIC index value was calculated according to Equation (2):

$$\text{HIC index} = \frac{(\%\text{R}_{\textit{butyl}}) + (\%\text{R}_{\textit{phenyl}}) + (\%\text{R}_{\textit{octyl}})}{300\%} \quad (2)$$

In the denominator, the 300% value represents the theoretical case of 100% retention on each column ideally achieved by a particle with maximum hydrophobicity. HIC index analysis was performed using a one way ANOVA comparison with a post-hoc Tukey test. p Value < 0.05 was considered to be statistically significant.

In vivo studies

Male BALBc mice (6–8 weeks old, ~22 g; Charles River, UK) were used for *in vivo* pulmonary administration. All experiments were in accordance with the UK Home Office regulations and approved by the local ethics committee. Animals were divided in to six groups ($n = 5$–7) for treatment with either the vehicle control (5% dextrose; DEX) or nanoparticle suspensions. Dosing was spread across four different dates, with vehicle controls (1–2 animals) dosed at every session and 2–4 animals from different nanoparticle groups treated on a rotational basis. A theoretical nanoparticle surface area dose of 220 cm^2 per instillation was chosen for study as this has been shown to induce a moderate inflammation in selected literature reports (Donaldson et al., 2000; Duffin et al., 2007). Particle surface area doses were calculated from the hydrodynamic diameter of the particles, assuming a density of ca. 1 g/cm^3 for PVAc nanoparticles and 0.96 g/cm^3 for LNC (estimated from the density of the oil which is the main constituent), and equated to ~200 µg nanoparticles per lung for the smaller LNC50 and PS systems and ~500 µg nanoparticles per lung for the larger LNC150, PVAc60 and PVAc80 systems. All suspensions were prepared in dextrose 5% w/v to ensure isotonicity and colloidal stability and a 5% w/v dextrose solution was used as the vehicle control ofr all experiments.

Prior to i.t. dosing, animals were anaesthetised by inhaled isoflurane (1–3%) and maintained with an intraperitoneal injection of 100 mg/kg ketamine mixed with 20 mg/kg xylazine in 0.1 mL saline. This combination of tranquiliser/dissociative yielded a moderate level of anesthesia for 15–20 min, as assessed by paw pinch withdrawal reflex. Mice were suspended at a 45° angle by their upper incisors and nanosuspensions (50 µL) were administered as a coarse aerosol into the lungs using a Penn Century Microsprayer® aerosoliser (Penn-Century Inc., Wyndmoor, PA). Animals were kept warm post-treatment with a heat lamp, then returned to their cages when ambulatory (<15 min). This administration method was chosen as it has been shown to achieve a more homogenous distribution of liquids into the lungs compared with conventional bolus i.t. instillation techniques (Bivas-Benita et al., 2005).

Bronchoalveolar lavage fluid analysis

Mice were euthanised via terminal anesthesia with urethane (2 mg/g i.p., Sigma Chemical Co.) 24 h after nanoparticle administration and a cannula was inserted into the exposed trachea. The lungs were lavaged with three aliquots (0.5 mL) of sterile saline that was recovered through the cannula. The total number of cells in the cellular fraction of the lavage was counted with a Neubauer haemocytometer (Fisher Scientific, Loughborough, UK). Differential cell counts were performed using cytospin preparations (i.e. 100 µL BAL cellular fraction centrifuged at 300 g for 1 min using a Shandon Cytospin 2 (Shandon Southern Instruments, Sewickley, PA) at room temperature). Cells were stained with Diffquick® (DADE Behring, Marburg, Germany) and a total of 200 cells were counted to determine the proportion of neutrophils, eosinophils and mononuclear cells using standard morphological criteria. Eosinophils were not observed in any of the samples and are not reported. It was assumed that at the time point studied, the mononuclear cell population consisted primarily of resident alveolar macrophages and therefore lymphocyte numbers were not investigated. The presence of erythrocytes in BAL was used as an indication of tissue damage. To evaluate this, the number of erythroctyes present within a single representative field of view (40× magnification) of a cytospin preparation were counted.

Cytokines present in the BAL supernatant were quantified using a murine 7-plex pro-inflammatory cytokine assay (MSD®96-Well Multi-Spot Cytokine Assay; Meso-Scale Discovery, Gainsborough, MD) coupled with an MSD reader, which measures cytokine content via electrochemiluminesce. Of the seven cytokines analysed (IFN-γ, IL-1β, IL-6, IL-10, IL-12p70, CXCL1 (KC/GRO/CINC), and TNF-α), IL-10 and IL-12p70 were below detectable limits and were therefore not reported. As a further measure of tissue integrity, total protein levels in BAL were quantified using a Quick Start™ Bradford Protein Assay (Bio-Rad, Hemel Hempstead, UK) according to manufacturer's instructions. A two-sided Mann–Whitney test was used for the comparison of BAL neutrophil counts, cytokine and protein levels in all the samples exposed to nanoparticles compared to the vehicle control (5% dextrose). p Value < 0.05 was considered to be statistically significant.

Sample preparation and UPLC-Q-ToF analysis

Extra pure formic acid and LC-MS grade acetonitrile (ACN) and water were purchased from Fluka (Sigma-Adrich). Aliquots (400 µL) of BAL fluid samples (DEX, $n = 6$; LNC50, $n = 7$; LNC150, $n = 6$; PVAc60, $n = 5$; PVAc80, $n = 7$; and PS, $n = 7$) were transferred to clean tubes and evaporated to dryness at 100 °C. Samples were reconstituted in 100 µL of 50:50 (v:v) purified water containing 0.1% formic acid and ACN containing 0.1% formic acid. Samples were vortexed for 1 min at room temperature. Quality control samples ($n = 5$) consisted of samples without a pre-concentration step. These were used to verify retention time and mass during the duration of the analytical run (Whiley et al., 2012). Samples were run on a Waters Acquity coupled to a Waters Xevo QTOF-MS. The UPLC was performed on a Waters ACQUITY UPLC™ system, equipped with binary solvent delivery manager, sample manager and quadrupole time of flight mass spectrometer. Parameters were as listed: chromatographic column (Garcia-Perez et al., 2010) UPLC-BEH C18, 2.1 × 100 mm (Waters Corporation, Milford, MA); mobile phase A: H_2O (0.1% formic acid) and B: ACN (0.1% formic acid); gradient analysis: 90% A and 10% B isocratic for 0–5, 5–20 min gradient to 100% B, 20–25 min isocratic 100% B; flow rate: 0.5 mL/min; pressure circa 9000 psi. The MS was operated in the positive ion mode with a capillary voltage of 2.7 kV and a cone voltage of 50 V. The desolvation gas flow was 490 L/h at a temperature of 300 °C and the cone gas flow was 10 L/h. A source temperature of 100 °C was used. All analyses were acquired using the lock spray to ensure accuracy and reproducibility; leucine enkephalin was used as lock mass (m/z 556.2771 Da) at a concentration of 200 ng/mL and a flow rate of 10 µL/min. Data were collected in the centroid mode with a lock spray frequency of 11s over the mass range m/z 50–850 with an acquisition time of

Table 1. Composition and physicochemical properties of the five organic nanoparticles studied.

	Lipid nanocapsules		Polymeric nanoparticles		
Abbreviation	LNC50	LNC150	PVAc60	PVAc80	PS
Nanoparticle core	90% TG	93% TG	99% PVAc60	96% PVAc80	Polystyrene
Nanoparticle stabiliser	0.3% PEG-HS 9.3% PC	0.2% PEG-HS 6.5% PC	0.8% PVA	3.8% PVA	Undisclosed
Diameter (nm)	40 ± 3	143 ± 2	160 ± 7	165 ± 7	54 ± 4
PDI	0.14 ± 0.02	0.32 ± 0.42	0.13 ± 0.03	0.08 ± 0.03	0.04 ± 0.02
ζ (mV)	−7 ± 4	−4 ± 1	−3 ± 1	−4 ± 1	−25 ± 5
Stability in H_2O (4 °C)	>4 weeks	>4 weeks	>4 weeks	>4 weeks	>4 weeks
Stability in HBSS (37 °C)	>24 h	>24 h	>24 h	Immediate aggregation	Immediate aggregation

PEG-HS = polyethylene $glycol_{660w}$-(15)-hydroxystearate; PC = soy lecithin; TG = medium chain triglycerides; PVA = polyvinyl alcohol; PVAc = polyvinyl acetate; PDI = polydispersity index; ζ = zeta potential.

250 ms, inter-scan delay of 50 ms. The chromatograms were obtained by injecting 4 µL. Sample sequences were assembled in blocks with blanks every seven injections to monitor for hydrophobic compound carry-over and QC samples were run to ensure analytical reproducibility. Identification of adenosine monophosphate (AMP) was made by first searching databases and then confirmation with MS-MS analyses of two AMP standard compounds (AMP 5′ and 3′ standards; Sigma-Aldrich, Dorset, UK).

UPLC-Q-ToF data analysis

UPLC-Q-ToF data were analysed using Mass Lynx version 4.1, published by Waters Corporation, Milford, MA, and exported to SIMCA-P™ software version 11.5, published by Umetrics AB, Umeå, Sweden. Data comprised 38 BAL samples, seven blanks and 4753 variables per chromatogram (each variable was a retention time and m/z). Models were created by normalising to total chromatogram area and scaling variables to *pareto* in all principal component analyses (PCA), orthogonal partial least squares discriminant analyses (OPLS-DA) and partial least squares (PLS) analyses. PCA was used to assess quality controls and identify outliers in the groups. PLS detects the variation in the fingerprint data as a whole (x-block) and compares it with variation patterns in the metadata (e.g. discrimination, hydrophobicity, neutrophilia or cytokine levels; y-block). PLS divides the analysis into two parts: one part models the covariation between the fingerprint patterns (R^2X% is the percentage correlation of the metabolite fingerprints or goodness-of-fit) and the second part models correlation to metadata (R^2Y%). The Q^2 (%) or goodness-of-prediction value expressed the prediction power of the model and is the output of a seven-fold cross-validation. Two to three components were calculated for each model. Models were cross-validated independently by 100-fold scrambling and a CV-ANOVA test. Only features that showed high correlation to either treatment group were considered for identification and semi-quantification.

Metabolite identification was achieved by database searching of in-house libraries and the Human Metabolome Database (HMDB; now containing 40 000 metabolite entries) for standard compounds and their molecular fragmentation pattern (Whiley et al., 2012; Xiayan & Legido-Quigley, 2008). A two-sided Mann–Whitney test was used to assess significance of AMP measurements for all samples exposed to nanoparticles compared to the vehicle control.

Results

Manufacture of organic nanoparticles with varying surface hydrophobicity

Two discrete size classes of nanoparticles (~50 and 150 nm) were included in the study (Table 1). The sizes were dictated by the chemical composition and the manufacturing techniques used to produce the nanoparticles. The nanoparticles studied did not exhibit a pronounced surface charge and all nanoparticles were physically stable in purified water for up to four weeks. LNC50, LNC150 and PVAc60 retained their original size in Hank's buffered saline (HBSS) at 37 °C for over 24 h, but PVA80 and PS50 aggregated immediately upon addition to HBSS (Table 1).

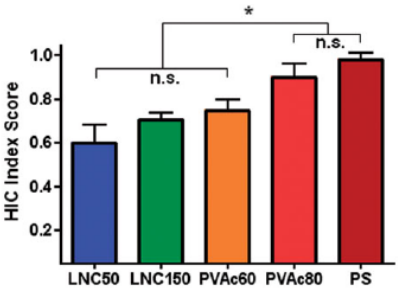

Figure 1. Quantitative evaluation of nanoparticle surface hydrophobicity expressed as the HIC index score of five nanoconstructs. Values represent the mean ± standard deviation of $n = 3$ individual nanoparticle batches. *$p < 0.05$.

The surface hydrophobicity of the nanoparticles was quantified using a HIC index scale (zero = hydrophilic, 1.00 = hydrophobic). The nanoparticles studied spanned the upper 50% of the HIC index scale, ~0.60–0.96 (Figure 1). PS beads exhibited a nearly maximal hydrophobicity (0.96 ± 0.03) and therefore served as an excellent reference material. The other nanoparticles used in the study exhibited a surface hydrophobicity rank order of LNC50 < LNC150 < PVAc60 < PVAc80. Statistical analysis of the HIC index values revealed two major groupings, lower surface hydrophobicity particles (LNC50, LNC150 and PVAc60; HIC index 0.60–0.80) and high surface hydrophobicity particles (PVAc80 and PS; HIC index > 0.85).

Respiratory toxicity of high surface hydrophobicity nanoparticles

Neutrophilia and elevated pro-inflammatory cytokine levels were observed at significant levels in BAL for the two high hydrophobicity nanoparticle treatments, PVAc80 and PS (Figure 2a, b, and d). The moderate inflammation induced in response to PS nanoparticles was consistent with benchmark studies (Dailey et al., 2006; Donaldson et al., 2000; Duffin et al., 2007). Total protein levels in BAL from nanoparticle treatment groups were not significantly different from the dextrose vehicle control or

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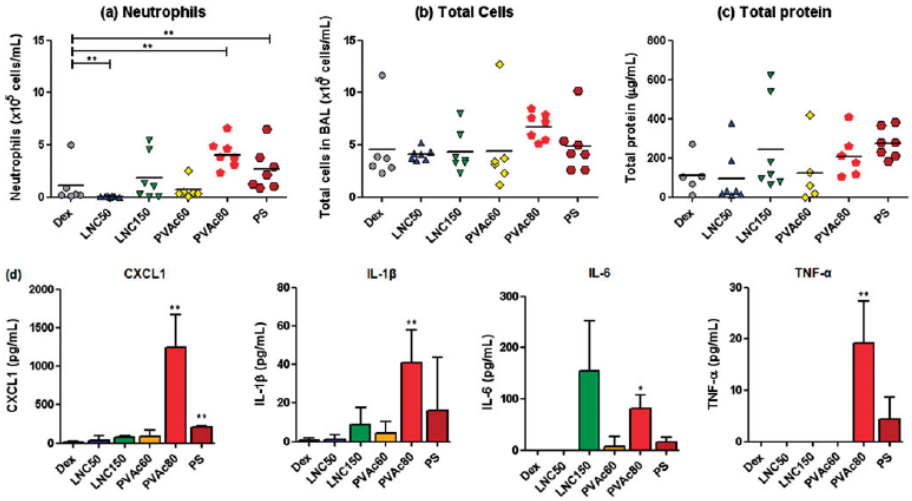

Figure 2. Assessment of respiratory toxicity 24 h post intratracheal administration of five nanoparticles based on (a) neutrophil counts, (b) total cells, (c) total protein levels and (d) pro-inflammatory cytokine content in BAL fluid ($n = 5$–7 individual animals per group). *$p < 0.05$, **$p < 0.01$.

each other (Figure 2c). Trends in the protein data, which also correlate with increase in BAL erythrocyte numbers (Supplementary Figure S1a) suggest that high hydrophobicity nanoparticle treatment, especially PS exposure, generally resulted in higher BAL protein levels indicative of possible tissue damage, and the lack of statistical significance may result from the variability in the vehicle control group.

The high variability in both the inflammatory profile and BAL total protein content of the vehicle control (5% dextrose) compared with untreated controls and animals administered 0.9% saline vehicle via an oral aspiration technique (Supplementary Figure S1b) suggests that Microsprayer® administration may be responsible. Careful analysis showed that variability occurred randomly and potential outliers could not be correlated with factors such as animal batch, date of experiment or experience of the operator with the Microsprayer® administration technique. Furthermore, including putative outliers in the study allowed us to examine whether their fingerprints showed metabolite patterns indicative of toxicity (i.e. similar to positive control profiles).

Nanoparticle treatment results in significant differences in BAL metabolite fingerprints

In this study, a total of 38 BAL fluid samples from the six treatment groups (vehicle control, nanoparticles, and quality control samples) were investigated. The fingerprints acquired consisted of chromatograms from which 4753 molecular features were extracted. In a preliminary analysis using PCA, quality controls were superimposable verifying analytical reproducibility. OPLS-DA was used to compare the fingerprint profiles of vehicle and PS- treated groups (Figure 3), which acted as negative and positive controls, respectively (Brown et al., 2001; Dailey et al., 2006; Duffin et al., 2007). The analysis discriminated between PS nanoparticle treatment and the vehicle control ($p = 0.041$), indicating that different metabolites dominated the fingerprints of lungs exposed to nanoparticles compared to those that were not. Five molecule masses (*m/z* 331.20, 284.95, 188.12, 182.18 and 174.10 Da) were identified from the model as unknown metabolites having the highest correlation (0.98 to 0.78) with PS exposure (elevated concentrations found in PS-treated group compared to vehicle control). These five molecules did not correspond to any of the known accurate masses in metabolite databases. In the case where molecular masses identified in a metabolomics study do not match reported masses and fragmentation patterns in databases, the process of identification requires isolation of the metabolite from the biological sample, utilisation of a variety of analytical approaches to solve the identity of the molecule, and finally MS-based verification using a pure reference compound. In many current studies, researchers will use the mass of unidentified metabolites as a biomarker until the tedious process of metabolite identification is completed.

OPLS-DA was also used to compare the BAL fingerprint profiles of animals exposed to lower hydrophobicity (LNC50, LNC150 and PVAc60) versus high hydrophobicity (PVAc80 and PS) nanoparticles (Figure 4). The analysis discriminated between the two HIC-index groupings ($p < 0.0001$). Interestingly, all five unknown metabolites associated with PS nanoparticle exposure (*m/z* 331.20, 284.95, 188.12, 182.18 and 174.10 Da) were positively correlated in this analysis (correlation = 0.83 to 0.50; Figure 4) suggesting that these molecules are interesting candidates for targeted studies to investigate their potential as biomarkers of respiratory toxicity resulting from high hydrophobicity nanoparticle exposure.

Partial least squares (PLS) analysis was performed to analyse the incremental relationship between nanoparticle hydrophobicity, inflammatory outcomes and the BAL fingerprints. Total protein levels in BAL were excluded from PLS analysis due to the lack of statistically significant differences between groups. PLS analysis metrics for covariance of fingerprint data and nanoparticle HIC index score, neutrophil counts or BAL CXCL1 concentration are listed in Table 2, while the PLS score plot correlating covariance of fingerprint data with HIC index score is presented in Figure 5. A substantial correlation between metabolite fingerprints and measures of lung inflammation (neutrophils and CXCL1) was

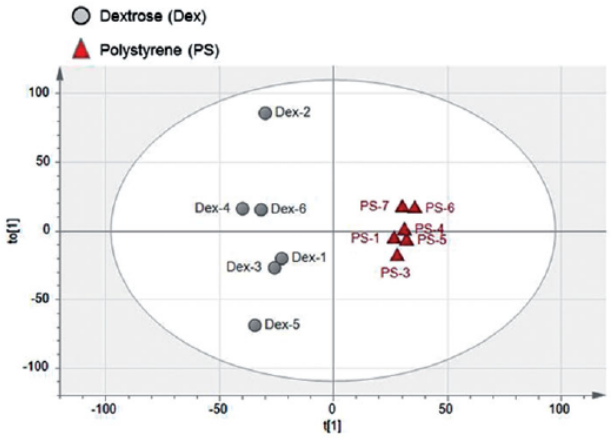

Figure 3. OPLS-DA scores plot of the vehicle control (5% dextrose solution) and positive control (PS nanoparticle exposure) showing group separation ($p = 0.041$).

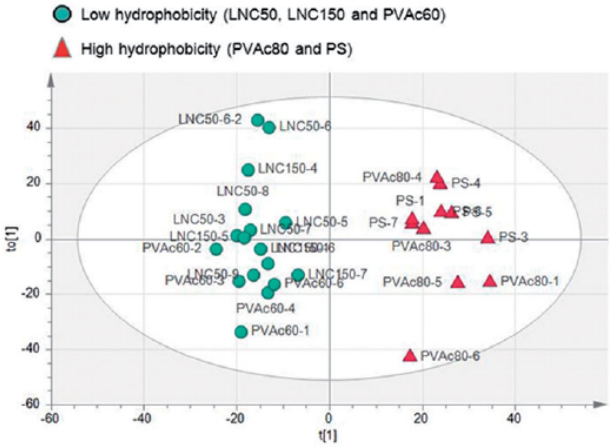

Figure 4. OPLS-DA scores plot of the lower surface hydrophobicity nanoparticle treatments (LNC50, LNC150 and PVAc60; HIC index: 0.6–0.80) and high surface hydrophobicity nanoparticle treatments (PVAc80 and PS; HIC index: >0.85) showing group separation (p value<0.0001).

Table 2. PLS analyses to assess correlations between BAL small molecule fingerprints and nanoparticle hydrophobicity (HIC index), neutrophil count or BAL CXCL1 concentration.

	HIC index	Neutrophils	CXCL1
R^2X (%)	28	28	29
R^2Y (%)	98	97	98
Q^2 (%)	66	65	68
p Value	0.001	0.01	0.04

The R^2X, R^2Y and Q^2 values are provided for each model in percentage.

indicated by the Q^2 values of 65 and 68%, respectively. The model for covariance between metabolite fingerprints and nanoparticle hydrophobicity showed a similar predictive Q^2 value of 66%, indicating a relationship between increasing nanoparticle hydrophobicity, lung inflammation and metabolite fingerprint models.

Elevated AMP levels induced by nanoparticles possessing high surface hydrophobicity

Analysis of the features that governed the correlation between metabolite fingerprint and HIC index identified a retention time and mass of interest. Using standard compounds and fragmentation patterns (Esther et al., 2008b), AMP (m/z 348.06 Da with a main fragment observed at 136.06 Da) was identified as a signature molecule associated with the effects generated in the lungs by nanoparticle of increasing hydrophobicity. The loading plot of the PLS model is provided in the Supplementary Figure S2, showing all metabolite data featured in the model. AMP is one of the major metabolites identified in the loading plot as the driving the separation of groups according to HIC index value. The black arrow in the PLS score plot (Figure 5) indicates that samples located along the direction of the arrow contained higher levels of AMP.

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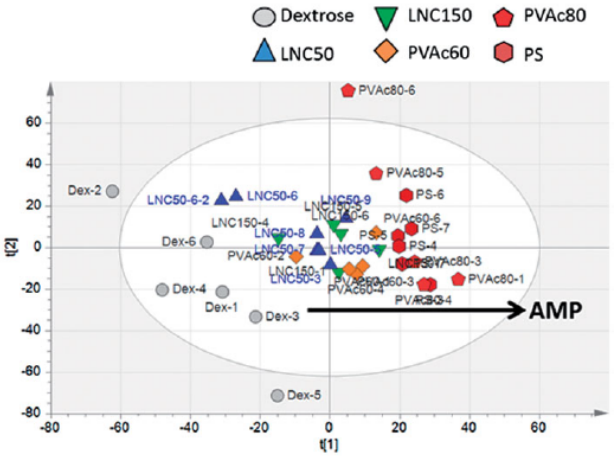
Figure 5. PLS scores plot showing the relationship between individual nanoparticle fingerprints and HIC index values. The figure also shows the direction of an identified metabolite, AMP, which follows the trend of increased nanoparticle-induced toxicity.



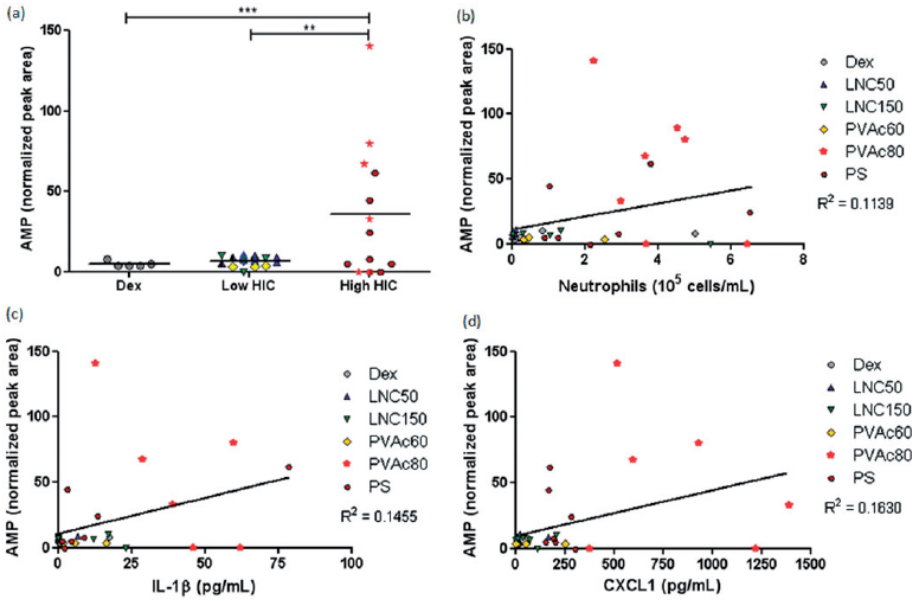


Figure 6. (a) AMP levels from vehicle controls, low hydrophobicity nanoparticle treatments (HIC index: 0.60–0.80) and high hydrophobicity nanoparticle treatments (HIC index: >0.85). ** $p<0.01$, *** $p<0.001$. AMP levels in each BAL sample (peak area normalised to total fingerprint area) are also plotted against paired values for (b) the number of neutrophils, (c) CXCL1 levels in BAL and (d) IL-1β levels in BAL.

AMP was measured in all the chromatograms and validation of the molecule identity was performed with the pure compound (Supplementary Figure S3 showing MS-MS AMP identification). AMP levels were only significantly elevated in high HIC nanoparticle treatment groups compared to both low HIC ($p<0.01$) and vehicle ($p<0.001$) treatment groups (Figure 6a). Scatter plots depicting paired values of AMP peak areas against neutrophil numbers, total protein levels and individual pro-inflammatory cytokine levels revealed significant correlations of elevated AMP with neutrophil counts (Figure 6b), CXCL1 levels (Figure 6c) and IL-1β levels (Figure 6d). Interestingly, the outlier in the dextrose vehicle

control group did not show highly elevated AMP levels in this analysis.

Discussion

The aims of this study were twofold: (1) to assess the impact of high versus low nanoparticle surface hydrophobicity on lung toxicity and (2) to apply a non-targeted metabolomics strategy to investigate whether organic nanoparticle exposure would result in significantly differentiated BAL metabolite profiles and to identify putative markers of nanoparticle exposure. Using conventional measures of pulmonary inflammation and tissue damage, it was demonstrated that nanoparticles of different composition exhibiting a high surface hydrophobicity (PVAc80 and PS) were pro-inflammatory with indications of tissue damage, while low hydrophobicity nanomaterials (LNC50, LNC150 and PVAc60) induced little to no toxicity according to these parameters. Hydrophobic nanoparticle surface chemistries may induce respiratory toxicity through more than one mechanism. For example, it has been shown that proteins and opsonins may adsorb more favourably onto a hydrophobic surface, promoting recognition, uptake and inflammatory signaling by phagocytic cells (Ruge et al., 2012; Singh & Lillard, 2009). A pertinent mechanism in this study may be particle aggregation in physiological fluids resulting in irregular surfaces, which have been associated with higher inflammatory potential than comparable smooth-surface particles (Vaine et al., 2013). Only the nanoparticles in the high surface hydrophobicity group (PS50 and PVAc80) aggregated in isotonic buffer at 37°C (Table 1), suggesting that this may occur to some extent *in vivo* and contribute to their enhanced toxicity profile.

In this study the effect of nanoparticle surface hydrophobicity on acute respiratory toxicity was established convincingly using conventional assays. Thus it was possible to undertake a non-targeted, phase one metabolomics study utilising the same BAL fluid samples. Non-targeted, NMR-based metabolomics screening has been used in a handful of studies to date to analyse intact tissues (lung, liver), as well as biofluids such as urine, serum and BAL from test animals after pulmonary, oral or intravenous exposure to different nanomaterials, including copper oxide, silica and titanium dioxide (Bu et al., 2010; Hu et al., 2008; Lei et al., 2008; Lu et al., 2011). Notably, Hu et al. (2008) examined the respiratory effects of i.t. administration of high dose silica nanoparticles one week and four months post-exposure. Metabolites identified by NMR as highly correlated with silica exposure (both in BAL and lung tissue) were primarily associated with cell membrane damage (elevated cholines and phosphocholine species), as well as elevated levels of hydroxyproline, indicative of the typical fibrosis development associated with silicosis (Hu et al., 2008).

In this study, five unidentified metabolites and AMP were identified as candidate biomarkers of high hydrophobicity nanoparticle exposure. While metabolite identification is of major importance for the design of hypothesis-driven mechanistic studies of toxicity, it must be emphasised that the identification process of unknown molecules from UPLC-MS chromatograms is not trivial, as current databases only hold metabolite mass and fragmentation pattern information on a small fraction of the estimated total number of possible metabolites (Whiley et al., 2012). AMP, in contrast, was identifiable from databases through its mass, fragmentation pattern and subsequent validation using a pure standard substance (Figures S3). It was observed that AMP levels in BAL fluid correlated with high nanoparticle surface hydrophobicity and acute lung inflammation. This observation was interesting as, in contrast to many putative biomarkers identified in non-targeted metabolomic screens, there is literature evidence to link AMP with known mechanisms of respiratory toxicity (Esther et al., 2008a, 2009; Patel et al., 2013; Schmidt & Tuder, 2010; Wolak, 2009).

A hypothetical basis for the source of elevated extracellular AMP in nanoparticle-induced respiratory toxicity is depicted in Figure 7. The schematic presentation illustrates the interplay between passive (e.g. via necrosis) and active (e.g. via neutrophil secretion) mechanisms of ATP/adenyl purine release into the extracellular fluid (Schmidt & Tuder, 2010). Cellular damage and release of intracellular contents into the extracellular environment is reported to be a putative source of elevated extracellular adenyl purines in conditions like acute lung injury

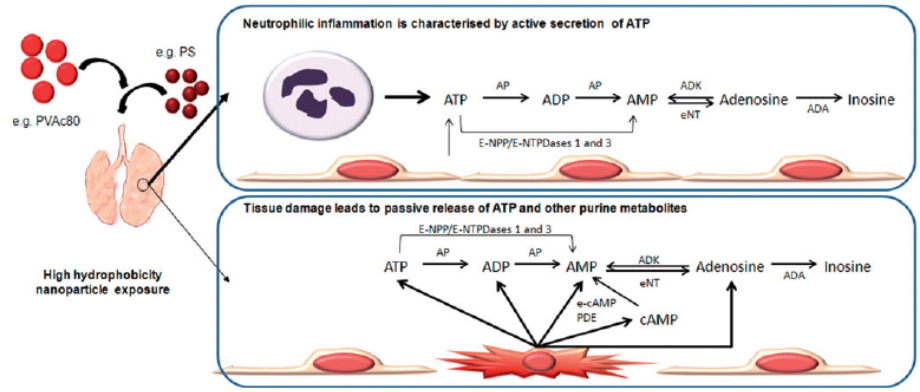


Figure 7. An illustration of the interplay between passive (e.g. via necrosis) and active (e.g. via neutrophil secretion) mechanisms of ATP/adenyl purine release into the extracellular fluid. The contribution of neutrophils and/or tissues to elevated purine levels in response to the administration of high hydrophobic surface nanoparticles is currently speculative. The simplified diagrams of extracellular ATP metabolism are based on information contained in (Barletta et al., 2012; Blackburn et al., 2009; Burch & Picher, 2006; Jackson et al., 2009; Robson et al., 2006; Schmidt & Tuder, 2010). E-NPP = Ecto-nucleotide pyrophosphatase/phosphodiesterases; AP = alkaline phosphatases; E-NTPDase = ecto-nucleoside triphosphate diphosphohydrolases; eNT = ecto-5′-nucleotidase; ADA = adenosine deaminase; ADK = adenosine kinase; e-cAMP-PDE = ecto-cAMP-phosphodiesterase.

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(Schmidt & Tuder, 2010). Elevated extracellular levels of ATP activate the purine receptors, P_2X and P_2Y subtypes, which are expressed across a wide range of cell types in the lung and specifically promote chemotaxis, release of pro-inflammatory cytokines, actin mobilisation and enhanced phagocytosis, as well as respiratory burst events in neutrophils (Barletta et al., 2012; Jacob et al., 2013). Notably, P_2X stimulation is associated with activation of the NLRP3 inflammasome in dendritic cells and subsequent secretion of IL-1β (Schmidt & Tuder, 2010).

Activated neutrophils also secrete high quantities of ATP into the extracellular environment, thus propagating inflammation (Figure 7; Barletta et al., 2012; Jacob et al., 2013). The potent pro-inflammatory effects of extracellular ATP are therefore regulated by its rapid metabolism in the lung lining fluid to ADP, AMP and adenosine. The lung mucosal surface possesses four major classes of enzymes to metabolise ATP to adenosine: ectonucleotidase-s:ecto-nucleotide pyrophosphatase/phosphodiesterases (E-NPP: ATP → AMP), alkaline phosphatases (AP: ATP → ADP → AMP → adenosine) and ecto-nucleoside triphosphate diphosphohydrolases (E-NTPDases: ATP → AMP) and ecto-5′-nucleotidase (eNT: AMP → adenosine) (Burch & Picher, 2006; Button & Button, 2013; Picher et al., 2003, 2004; Robson et al., 2006). Currently, relatively little is known about the direct role of extracellular AMP in inflammation, except as an intermediate metabolite. Adenosine, in contrast, is known to be both a pro-and anti-inflammatory signaling molecule. For example, in neutrophils, adenosine binding to adenosine receptor subtypes A_1 and A_3 generally promotes pro-inflammatory responses, while A_{2A} and A_{2B} activation results in anti-inflammatory effects, such as reduction in pro-inflammatory cytokine release, phagocytosis and degranulation (Barletta et al., 2012; Blackburn et al., 2009; Reutershan, 2009). Thus, the regulation of neutrophil response in lung inflammation is controlled both by levels of extracellular signaling molecule levels, as well as differential purine and adenosine receptor expression patterns.

The use of elevated extracellular purine levels as a putative biomarker for disease-induced neutrophilic inflammation has been reported previously. ATP and AMP have been observed to be elevated in BAL fluid samples from cystic fibrosis (CF) patients compared to disease controls (patients with unrelated respiratory disease) as well as in exhaled breath condensate from CF patients versus healthy controls (Esther et al., 2008a, 2009; Patel et al., 2013; Wolak, 2009). Further, a highly significant correlation ($p < 0.0001$) between neutrophil counts and increases ATP and AMP was found for all samples irrespective of disease state, supporting the authors' hypothesis that elevated ATP and AMP were a product of the secondary neutrophilic inflammation rather than the primary pathology (Esther et al., 2008a). Interestingly, it was also observed that AMP levels were consistently higher than those of ATP (Esther et al., 2008a), which was speculated by the authors to arise from increased metabolism of ATP to AMP by E-NTPDases (present on both neutrophils and epithelial cell surfaces) combined with a lower capacity to convert extracellular AMP to adenosine during inflammation due to a relative lack of 5′-nucleotidase (eNT: AMP → adenosine) on the surface of infiltrating neutrophils.

It should be noted that while the studies described above were able to show a strong direct correlation between neutrophil numbers and AMP levels, as well as ATP levels (Esther et al., 2008a, 2009; Esther et al., 2008a; Patel et al., 2013; Wolak, 2009), our preliminary results did not reveal as high as a correlation between neutrophil numbers and normalised AMP peak areas, even within the high hydrophobicity nanoparticle treatment group (Figure 6b). This discrepancy might be explained by the fact that the studies cited above all used optimised protocols to specifically measure ATP and AMP levels in their samples. This was representative of a targeted, phase two approach in the biomarker development pathway. In contrast, the analytical protocol used in the current study was designed to maximise the detection of the largest number of unknown metabolites, and may not have favoured purine detection. In fact, Esther et al. (2008b) reported that higher polarity nucleotide species, such as ATP, can be sensitive to low pH mobile phases such as those used in this study (e.g. 0.1% formic acid pH 3–5) and this can have an effect on the limits of detection of the metabolite (Esther et al., 2008b). AMP, a less polar metabolite, is more easily detected in a low pH mobile phase, but may still require further optimisation. Thus, it should be emphasised that targeted, phase two studies using optimised analytical conditions to assess concurrent levels of ATP, ADP, AMP, cAMP and adenosine with a longitudinal experimental design are required to shed further light on both origin and contribution of extracellular purines in nanoparticle-induced acute respiratory toxicity.

Conclusions

This study has demonstrated that nanoparticles with a high surface hydrophobicity induce acute respiratory inflammation when administered into the lungs of mice. The inflammation was characterised by significant neutrophilia, elevated levels of pro-inflammatory cytokines, evidence of increased tissue damage and significantly altered metabolite fingerprints in BAL fluid. AMP concentration in BAL was found to correlate with the degree of inflammation 24 h after the administration of the nanoparticles, the time point of peak neutrophilic response. This provides an interesting addendum to the hypothesis that elevated extracellular purines in the lungs are candidate biomarkers for neutrophilic lung inflammation. This is the first study to our knowledge to demonstrate elevated AMP in an acute response to the pulmonary administration of nanoparticles.

The non-targeted metabolomics screen also yielded five unidentified metabolites that were highly correlated with exposure to toxicity-inducing nanoparticles. The identities of these metabolites may be elucidated in future studies by isolation of molecules and LC-MS techniques combined with NMR analysis. Similar to AMP, the identities of these molecules may shed further light on individual mechanisms of respiratory nanotoxicity and they have the potential to expand meaningfully the range of nanotoxicity biomarkers available to researchers today.

Declaration of interest

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Supplementary material available online

Supplementary Figures S1–S3