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Analysis of glycerophosphoinositol by liquid chromatography–electrospray ionisation tandem mass spectrometry using a β-cyclodextrin-bonded column

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Abstract

Glycerophosphoinositol (GroPIns) has been demonstrated to have important roles in many intracellular regulatory processes. GroPIns has been analysed for many years by anion-exchange HPLC after radiolabelling of cells in culture, but no method has been developed, to our knowledge, for the direct detection and quantitation of the unlabelled compound in such biological samples. Here is reported a liquid chromatography–tandem mass spectrometry (LC–MS/MS) method for the direct quantitative analysis of GroPIns that can indeed be applied to cell extracts. Analyses were performed on a β-cyclodextrin-bonded HPLC column using a binary mobile phase of acetonitrile and 20 mM ammonium formate in water, which allowed direct on-line detection by tandem mass spectrometry in negative electrospray ionisation (ESI) mode. The method was applied to the quantitative analysis of GroPIns in selected rat cell lines after a two-phase acid extraction of cultured cells using external calibration. The potential matrix signal suppression effects were investigated by the parallel quantitation of GroPIns in extracts of selected cultured cell lines with both external calibration and the standard additions method. The accuracy data obtained demonstrated the feasibility of external calibration, so allowing a simpler and less time-consuming approach than that of the standard additions method.

Keywords: Glycerophosphoinositol; Cultured cells

1. Introduction

Inositol phosphate chemistry and biochemistry have been widely investigated over the past two decades due to the important and ubiquitous roles of inositol-containing compounds as cellular signalling substances in many regulatory processes, such as signal transduction, membrane transport and secretion. Among these, glycerophosphoinositol (GroPIns; Fig. 1) and its phosphorylated derivatives have proven to be important in modulating cell proliferation and G-protein-dependent activities, such as adenylyl cyclase and phospholipase A_2 [1]. Moreover, increased levels of GroPIns have been observed in a number of transformed cells, leading to the proposal that GroPIns cellular levels can be used as a biochemical marker of malignant cellular transformation [2–5].

In order to better elucidate the role of GroPIns in these cellular transformations, a specific and sensitive method is needed for its quantitative analysis at intracellular levels. A number of reviews about methods for analysis of the inositol phosphates, generally including GroPIns, in cellular systems [6–8] and also for application in nutritional studies [9] have been published. The technique used for detection and quantitation of these compounds depends greatly upon the system employed for their separation. For many years, GroPIns has been commonly analysed with the inositol phosphates by anion-exchange HPLC [4,5,10,11] using gradients of aqueous mobile phases with high concentrations of buffer solutions and employing radiolabelling techniques or inorganic phosphate analysis as detection and quantitation systems. An amino-phase column has also been used to separate GroPIns and other inositol phosphates, with the

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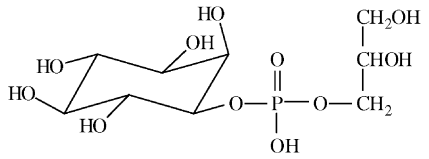

Fig. 1. Chemical structure of GroPIns (Mr 334.2).

use of an acetate buffer in the mobile phase [12]. Furthermore, a HPLC method using a reversed-phase column and a surfactant-containing mobile phase, thus resembling an ion-pairing mechanism, has been described [13]. Hence, investigations into inositol phosphate and glycerophosphoinositol biological pools are commonly performed by radiolabelling with [^{3}H]-*myo*-inositol, with the subsequent measuring of the formation of radiolabelled products by on-line or off-line liquid scintillation counting. However, this technique has the main disadvantage of being a multi-step procedure that only measures the true intracellular levels of these compounds if the radiolabelling is taken to isotopic equilibrium, which is often difficult to achieve, and if the analysis follows acute stimulation/modulation conditions.

On the other hand, HPLC analysis of non-radioactive GroPIns with on-line detection has always been a problem since, as can be deduced from its chemical structure (see Fig. 1), it is a polar compound which does not contain any UV-Vis or fluorescence-detectable group. Separation systems other than HPLC, as well as detection techniques other than radiolabelling, have been reported for inositol phosphates, and among these mass spectrometry detection with different ionisation sources have been described in a number of reports [14–16]. However, no method is available with on-line detection of unlabelled GroPIns. Electrospray ionisation mass spectrometry (ESI-MS) allows a direct and specific detection from biological matrices and has represented a significant advance in the analysis of intact phospholipids, allowing a greater sensitivity than that previously achieved by other ionisation techniques. Application of ESI-MS to phospholipids has recently been reviewed in detail [17] and the use of this approach has also recently been reported for phosphoinositide profiling in complex lipid mixtures [18]. Following the importance of glycerophosphoinositol lipids as constituents of membranes and precursors of signalling molecules, ESI-MS was applied early to the analysis of this class of phospholipids in both positive and negative ion modes [19]. The negative ion mode is, however, typically used to analyse glycerophosphoinositol lipids since a greater abundance of molecular ions is obtained. ESI tandem mass spectrometry of glycerophosphoinositol lipids in negative ion mode with elucidation of their fragmentation pathways has also been investigated in detail [20]. Combined with HPLC, mass spectrometry has been applied to the separation and identification of alkylacyl glycerophosphoinositol molecular species in human and bovine erythrocytes [21]. However, when HPLC is coupled to ESI-MS for analysis of deacylated glycerophosphoinositol lipids (i.e. GroPIns and its phosphorylated derivatives), the chromatographic conditions commonly employed use highly concentrated and often non-volatile buffer solutions, and are thus no longer suitable for ESI-MS due to the consequent suppressed ionisation of target compounds or clogging of the source orifice. In order to overcome these problems of coupling to ESI-MS, a β-cyclodextrin-bonded column has proven to be an effective choice, as it can be operated under water/organic conditions with the use of volatile modifiers at low concentrations. This stationary phase has been successfully used for the HPLC analysis of a number of compounds, such as oligosaccharides [22,23], sulphonated aromatics [24] and amino acid racemates [25], allowing the separation of both enantiomeric forms and positional isomers. Furthermore, various phosphorylated carbohydrates have been analysed on a β-cyclodextrin-bonded column by HPLC with ESI tandem MS detection [26].

Here we describe a HPLC method using a β-cyclodextrin-bonded column with on-line negative ESI tandem MS that can provide a direct and sensitive quantitation of GroPIns levels in cell extracts. The analytical approach, using external calibration in water, has been evaluated in terms of matrix effects, and then of accuracy by direct comparison with the standard additions method using cell extracts as matrix. To our knowledge, this is the first method employing liquid chromatography coupled to ESI tandem MS for the analysis of GroPIns, providing a favourable alternative to the disadvantages of the radiolabelling method that is commonly used for this compound.

2. Experimental

2.1. Reagents and standards

Acetonitrile (HPLC grade) and ammonium formate (analytical grade) used for chromatographic analyses were both purchased from Carlo Erba Reagenti (Milan, Italy). Sterile deionised water, obtained from Laboratori Diaco Biomedicali (Trieste, Italy), was used for sample reconstitution and for chromatographic analyses. L-α-Glycerophospho-D-*myo*-inositol (GroPIns; 5 mg/ml in methanol:water, 1:1) was purchased from Sigma (St. Louis, MO, USA) as potassium salt of 98% purity. All media and products for cell culture were purchased from Sigma or Gibco BRL (Grand Island, NY, USA).

2.2. Equipment and analytical conditions

2.2.1. Liquid chromatography

Chromatographic analyses were performed at room temperature (20 ± 1 °C, unless otherwise stated) with a HPLC system consisting of two Perkin-Elmer series 200 micro-LC pumps (Norwalk, CT, USA) and using a CC 200/4 Nucleodex β-OH analytical column (200 mm × 4 mm, 5 μm) equipped with a CC 8/4 Nucleodex β-OH guard column

(8 mm × 4 mm, 5 μm), both purchased from Macherey-Nagel (Düren, Germany). Samples (20 μl) were automatically injected using a Perkin-Elmer series 200 autosampler (thermostated at 4 °C). The elution was carried out at the flow rate of 0.7 ml/min, with a mobile phase consisting of 20 mM ammonium formate aqueous solution (pH 6.3, solvent A) and acetonitrile (solvent B) according to the following gradient: from 20 to 56% solvent A in 12 min; the mobile phase was then returned to the initial conditions, thus allowing the system to re-equilibrate (at least 10 min). Analysis of standard GroPIns at column temperatures other than room temperature for the investigation of its double-peak profile were performed by using an external water bath, in which the analytical column was immersed, thermostated at 6 ± 1 °C with an Endocal refrigerated circulator (Neslab, Newington, NH, USA). Alternatively, a Gecko-2000 HPLC column heater (Cluzeau Info Labo, Sainte-Foy-La-Grande, France) was used for the analysis at the controlled temperature of 50 ± 1 °C.

2.2.2. Mass spectrometry

A Sciex API 365 triple quadrupole mass spectrometer (Toronto, Canada) was coupled to the HPLC system through a Sciex TurboIonSpray source operated in negative ESI mode. Instrument control, data acquisition and processing were performed using Masschrom 1.1.1 software (PE Sciex, Foster City, CA, USA) run on a Macintosh G4/400 computer (Apple, Cupertino, CA, USA). The mass spectrometer was initially calibrated using polypropylene glycol as standard (Applied Biosystems, Foster City, CA, USA), setting the resolution, as peak width at half height, in the range 0.7 ± 0.1 u.

Mass spectrometric parameters for GroPIns were optimised by direct infusion of the standard compound (100 μg/ml) in the stream of the mobile phase (50% solvent A, 0.7 ml/min) by using a "make-up" system and a model 11 syringe pump (Harvard Apparatus, South Natick, MA, USA) operated at the flow rate of 5 μl/min.

The analyses were performed by multiple reaction monitoring (MRM) mode, using the "precursor to product ion" pair m/z 333 → 153. The nebuliser gas flow (air) and the curtain gas flow (nitrogen) were set at 2.1 and 2.0 l/min, respectively. The pressure of collisionally activated dissociation (CAD) gas (nitrogen) was maintained at 0.273 Pa and the collision energy was 27 eV. The TurboIonSpray source was operated at 475 °C with the auxiliary gas flow (nitrogen) set at 8 l/min. The TurboIonSpray voltage was set at −5400 V and the orifice and ring voltages at −41 and −180 V, respectively. Dwell time was 400 ms.

Full scan MS analysis of standard GroPIns was performed by scanning the range m/z 50–750 with a step size of 0.1 u and a dwell time of 0.172 ms, and using the same parameters of MRM mode reported earlier for the TurboIonSpray, orifice and ring voltages, for the nebuliser, curtain and auxiliary gases and for the TurboIonSpray source temperature.

2.3. Cell culture and extraction

Control and Ha-*Ras*-transformed rat fibroblasts (FRT-Fibro and FRT-Fibro-Ha cells, respectively) and control and Ki-*Ras*-transformed rat thyroid (FRTL5 and KiKi, respectively) cells were cultured and extracted as described in detail in our preliminary report of the biological application of this LC–MS/MS method [27]. Lyophilised extracts were resuspended with 200 μl water just before analysis.

Similarly, GroPIns recovery in cell extracts for LC–MS/MS analysis was evaluated, as indicated in [27], with a number of different control and transformed cell lines (two Petri dishes of each cell type on selected days) after their spiking with a known amount of [^{3}H]-GroPIns (∼300,000 dpm). The GroPIns recovery through the cell extraction procedure was found to be 99.4% (R.S.D. = 3.2%, $n = 32$).

2.4. Standard solutions

The GroPIns standard solution purchased from Sigma was aliquoted, lyophilised and resuspended in water in order to obtain aliquots of the standard compound at the concentration of 2 mM. A stock solution of GroPIns at 100 μg/ml was prepared from the 2 mM aliquot by dilution in water. The 100 μg/ml stock solution was further serially diluted in water in order to obtain working standard solutions over the range 2.5–8000 ng/ml. All standard solutions were stored at −20 °C until their use.

2.5. GroPIns external calibration

The linearity of the external calibration method for GroPIns was evaluated by the analysis of five independent calibration curves ($n = 5$), consisting of nine concentration levels (2.5, 5, 10, 25, 50, 100, 250, 500, 1000 ng/ml working standard solutions). The linearity of each calibration curve was calculated by quadratic least-squares regression of analyte double peak areas versus concentrations. A weighting factor ($1/y$) was applied to each standard curve to determine slopes, intercepts and correlation coefficients (r). The range of the method was assessed as the interval of concentrations in which the correlation coefficient was ≥0.985 and precision and accuracy (calculated as described further) were ≤15%, except for the lower limit of quantitation (LLOQ), defined as the lowest quantifiable concentration for which precision and accuracy ≤20% were accepted.

After back-calculating analyte concentrations from each curve equation, precision was determined as the relative standard deviation (R.S.D., %) of all of the back-calculated values for each concentration level, and the accuracy was determined for each level as: [(mean calculated concentration − nominal concentration)/nominal concentration] × 100.

2.6. GroPIns standard additions

To investigate the influence of matrix effects on the ESI efficiency, and then to evaluate the accuracy of the external

Table 1
Linearity data for GroPIns using the standard additions method for selected rat cell lines and the external calibration method in water

	Standard additions[a]				External calibration[b]
	FRT-Fibro	FRT-Fibro-Ha	FRTL5	KiKi	
Concentration range (ng/ml)[c]	50.00–1000	500.0–8000	500.0–8000	100.0–2000	5.000–1000
Slope (mean ± S.D.)	164 ± 23	155 ± 26	147 ± 16	150 ± 18	154 ± 8
Intercept (mean ± S.D.)	6208 ± 157	153791 ± 23155	76697 ± 16654	25319 ± 3828	236 ± 86
r (mean ± S.D.)	0.9994 ± 0.0006	0.9994 ± 0.0002	0.9991 ± 0.0003	0.9989 ± 0.0007	0.9996 ± 0.0003

[a] $n = 3$ for each cell line.
[b] $n = 5$.
[c] For the standard additions samples: concentration range of GroPIns working standard solutions added to the lyophilised cell extracts; the range includes the non-spiked sample.

calibration in water, an analysis with the standard additions method was performed using as matrix the cell extracts of the two paired cell lines (control and *Ras*-transformed) FRT-Fibro/FRT-Fibro-Ha and FRTL5/KiKi, prepared as described in Section 2.3. Thus, these lyophilised cell extracts were resuspended with 200 μl of GroPIns working standard solutions in water at increasing concentrations selected in the range 50–8000 ng/ml, according to the expected endogenous GroPIns content in the extracts of each cell line (see Table 1).

The linearity of standard additions curves was evaluated for each cell line by the analysis of three independent calibration curves ($n = 3$), consisting of the non-spiked sample (extract resuspended with water) and five spiked samples (extracts resuspended with GroPIns working standard solutions, as described earlier). The linearity of each calibration curve was calculated by quadratic least-squares regression of analyte double peak areas versus concentrations, and the slopes, intercepts and correlation coefficients (r) for each curve were determined. These analyses of the standard additions samples were done in parallel with the external calibration curves in water to compare the quantitative results obtained for the examined lines using the external calibration with those using the standard additions method.

3. Results and discussion

3.1. Mass spectrometry

The full scan mass spectrum of GroPIns was initially obtained by direct infusion of the standard compound as aqueous solution through the ESI source. The negative ionisation pattern showed the deprotonated molecular ion $[M-H]^-$ as the main peak. Curtain and nebuliser gas flows and ionspray, orifice and ring voltages were optimised so as to maximise the $[M-H]^-$ signal. Fragmentation of GroPIns $[M-H]^-$ ion was then achieved. The product ion mass spectrum of GroPIns acquired under optimised fragmentation conditions (collision energy, CAD gas flow) of the deprotonated molecular ion is shown in Fig. 2. GroPIns ($[M-H]^-$ m/z 333) fragmentation resulted in the main product ion at m/z 153, corresponding to the loss of the inositol group (180 u) from the molecular ion. Ions at m/z 79 and 97, corresponding to PO_3^- and $H_2PO_4^-$ species, respectively, are indicative of the phosphate moiety. As a result of the product ion spectrum obtained, MRM detection of GroPIns was performed using the "precursor to product ion" transition m/z $333 \rightarrow 153$.

3.2. Liquid chromatography

Conventional LC methods used for anionic compounds such as GroPIns and its phosphorylated derivatives are based on anion-exchange or ion-pairing chromatography which prevent the use of specific MS detection. As an alternative, the β-cyclodextrin-bonded stationary phase has allowed us to analyse GroPIns using a binary mobile phase of acetonitrile and 20 mM ammonium formate in water, thus enabling its ESI-MS analysis with an absolute limit of detection (LOD), evaluated as the lowest detectable amount with a signal-to-noise ratio of 3, of 50 pg. As shown in Fig. 3, where representative LC-MRM chromatograms of standard GroPIns at different HPLC column temperatures are reported, chromatography resulted in two distinct peaks,

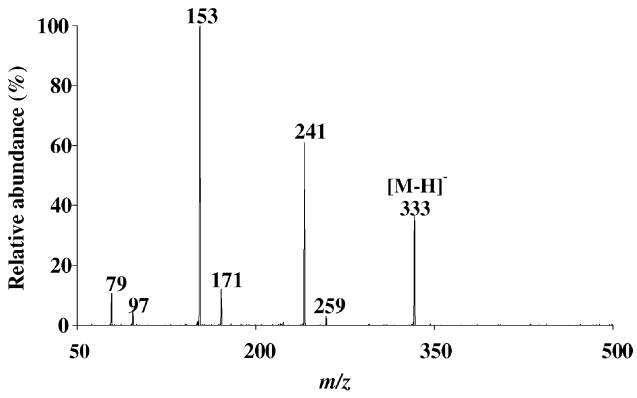

Fig. 2. Product ion scan mass spectrum of standard GroPIns (MS/MS of m/z 333) over the range m/z 50–500. The mass spectrum was acquired under optimised fragmentation conditions of the $[M-H]^-$ ion (see Section 2.2.2 for details).

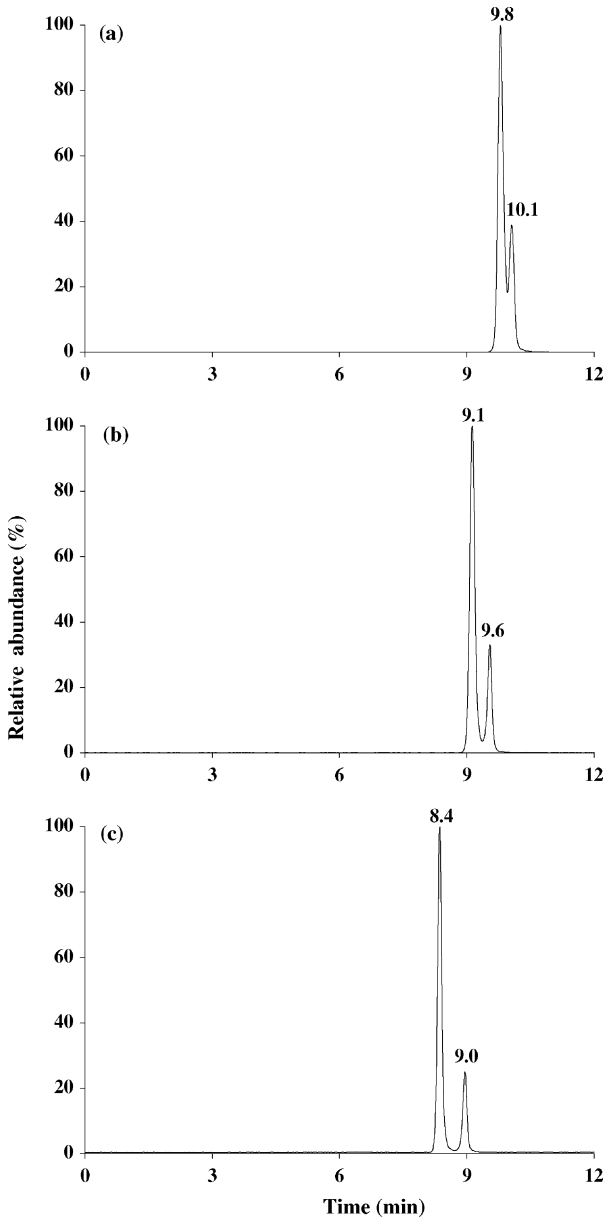

Fig. 3. LC-MRM chromatograms (*m/z* 333 → 153) of standard GroPIns at the HPLC column temperatures: (a) 6 °C; (b) room temperature (20 °C); (c) 50 °C. LC and MS conditions were as described in Sections 2.2.1 and 2.2.2.

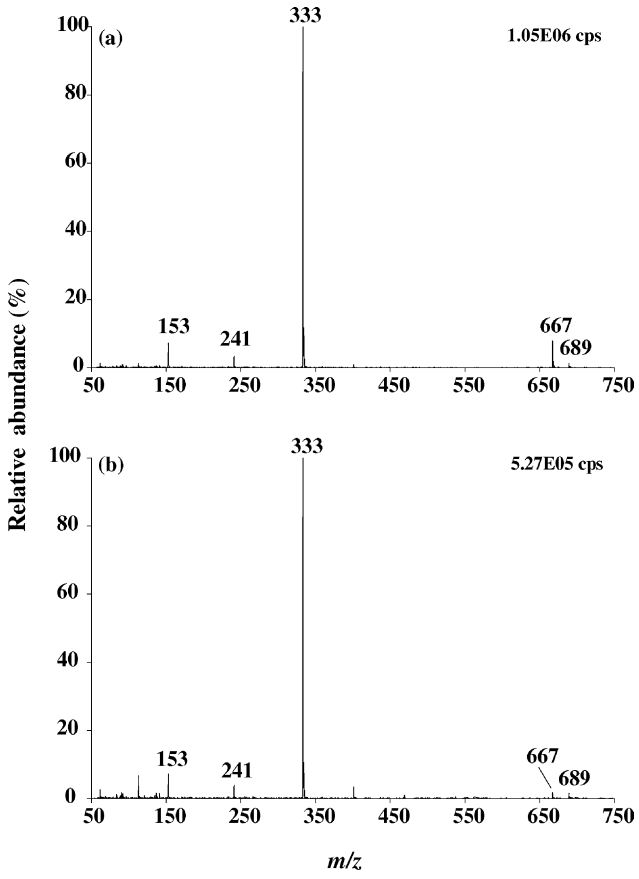

Fig. 4. ESI mass spectra over the range *m/z* 50–750 of the two individual GroPIns peaks obtained from the full scan MS chromatogram of the standard compound (100 μg/ml in water): (a) first eluting peak; (b) second eluting peak (see profiles of Fig. 3). LC and MS conditions were as described in Sections 2.2.1 and 2.2.2.

with an area ratio of ∼80:20. This same ratio was maintained also when analyses of standard GroPIns were performed at temperatures other than room temperature (20 °C, Fig. 3b), chosen in the allowed range of the column (6 °C, Fig. 3a; 50 °C, Fig. 3c), to determine if there was a potential temperature-dependent equilibrium between these two peaks. Identity of these two individual peaks of GroPIns was tested by full scan MS chromatography of the standard compound and the ESI mass spectra of the two peaks are shown in Fig. 4. The substantial identity of the two spectra clearly proves that both peaks are from the GroPIns compound. Moreover, the two peaks from GroPIns chromatography were also individually collected and re-injected soon after the end of the separation run, after being diluted in water so as to minimise their organic solvent content and thus to avoid modifications in their elution profiles due to an incomplete aqueous composition as compared to the chromatographed standard. Chromatography of both of the re-injected peaks resulted in identical (∼80:20) chromatographic profiles as seen for the standard compound (see Fig. 3b). The same ∼80:20 splitting was also obtained when the collected peaks were first lyophilised and then resuspended in water for re-injection about 2 h after collection. As a whole, these data thus suggest that the two peaks seen in the chromatography of GroPIns do not represent different optical isomers; however, we can hypothesise that different conformers relating to the *myo*-inositol moiety of the compound may prove to be responsible.

This method has been used to directly measure GroPIns levels in extracts from different rat cell lines using external calibration in water, after monitoring of a number of method parameters and evaluation of potential matrix signal suppression effects, as described in Sections 2.5 and 2.6, respectively. The sum of the two peak

areas detected for GroPIns was used for quantitation purposes.

3.3. GroPIns external calibration

GroPIns calibration curves in water showed excellent linearity in the calibration curve range 5–1000 ng/ml (data shown in Table 1). The lower limit of quantitation for GroPIns, assessed according to the criteria reported in Section 2.5, was 5 ng/ml. Good precision and accuracy results of this method for the quantitative analysis of GroPIns were obtained. The precision ($n = 5$) was below 6.1% over the entire concentration range, except at LLOQ (11.8%), while accuracy ($n = 5$) ranged from 0.9 to 3.0%.

3.4. GroPIns standard additions

The standard additions method was investigated in parallel with the external calibration in water for the quantitation of intracellular GroPIns levels in selected cell lines, as described in Section 2.6. This was to evaluate the potential influence of matrix effects on the LC–MS/MS analysis of GroPIns and then the accuracy of the external calibration method, calculated as relative to the standard additions method. The use of external calibration in water was considered as a favourable alternative to the standard additions method, thus avoiding time-consuming experimental steps in preparing cell extracts and in their spiking, which may be a limiting problem especially when a considerable number of different cell lines have to be analysed. Similarly, the use of an internal standard was initially considered; however, the lack of easy availability of stable isotopically labelled compounds, or of a different standard compound with suitable physico-chemical properties, not endogenously present in cells, made this approach not feasible.

As shown in Table 1, good linearity was obtained with the standard additions method for each of the four examined cell lines, with the correlation coefficient (r) above 0.9989 for all the cell lines tested.

3.5. Application to intracellular GroPIns measurements

The approach of external calibration was examined by investigation of the ESI process susceptibility to matrix signal suppression effects (see above). This was achieved by the parallel quantitation of GroPIns in extracts of selected cell lines with external calibration and with the standard additions method using extracts of those selected cell lines as matrix. Fig. 5 shows the extracted ion chromatograms of GroPIns from the analysis of extracts from two paired (control and *Ras*-transformed; FRT-Fibro/FRT-Fibro-Ha and FRTL5/KiKi) rat cell lines. The concentrations of GroPIns in these extracts are summarised in Table 2, where the results obtained from replicate analyses for each cell line with both the standard additions method and the external calibration method are reported. These results demonstrate

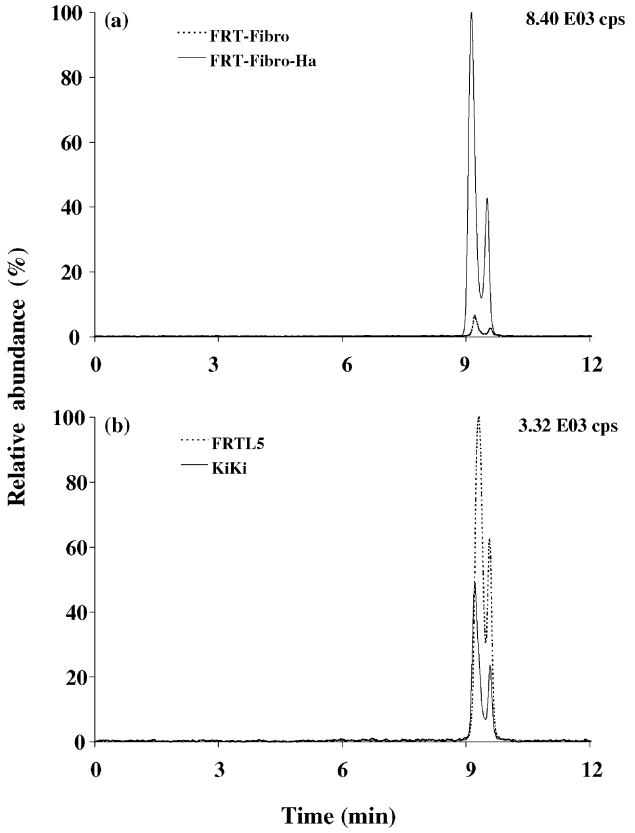

Fig. 5. LC-MRM chromatograms of GroPIns (m/z 333 → 153) after the two-phase acid extraction of cultured FRT-Fibro (control) and FRT-Fibro-Ha (Ha-*Ras*-transformed) cells (a), and FRTL5 (control) and KiKi (Ki-*Ras*-transformed) cells (b). Both peaks of each chromatogram represent the GroPIns compound. LC and MS conditions were as described in Sections 2.2.1 and 2.2.2. Concentrations of GroPIns in the extracts of these examined cell lines are summarised in Table 2, as mean values of replicate analyses.

the feasibility of external calibration in water for the quantitation of GroPIns in cell extracts as an alternative to the multi-step and time-consuming standard additions method, as its accuracy was estimated in comparison with the standard additions approach as being below 15% for all the cell lines tested.

The specificity of the MRM detection provides a very clean chromatographic profile with no interfering peaks from the cell extracts. This thus allows the sensitive determination of intracellular GroPIns concentrations (in combination with cell number and cell volume data), which can provide direct information as to the effects of *Ras* transformation on intracellular GroPIns levels. The implications of these effects on the potential use of GroPIns levels as a biochemical marker of malignant cellular transformation, following the application of this method to a larger number and variety of control and transformed cell lines, have been reported and discussed elsewhere as part of a specific analysis of the selective maintenance of the polyphosphoinositide pools in cells [27].

Table 2
Quantitative results for GroPIns in extracts from selected rat cell lines obtained by using the methods of the standard additions and external calibration in water

Cell line	Standard additions[a]		External calibration[b]		Accuracy (%)
	Mean calculated concentration ± S.D. (ng/ml)[c]	Precision (R.S.D., %)	Mean calculated concentration ± S.D. (ng/ml)	Precision (R.S.D., %)	
FRT-Fibro	38.1 ± 4.1	10.7	33.7 ± 1.6	4.8	−11.7
FRT-Fibro-Ha	996 ± 78	7.8	920 ± 92	10.0	−7.6
FRTL5	519 ± 73	14.1	460 ± 69	14.9	−11.3
KiKi	168 ± 12	6.9	146 ± 11	7.8	−13.6

[a] $n = 3$.
[b] $n = 5$.
[c] Calculated by extrapolation of the standard additions regression curve to the *x*-intercept.

4. Conclusions

In this study, we have described a LC–MS/MS method for the quantitative analysis of GroPIns in cell extracts. The method allows the sensitive quantitation of GroPIns levels by the use of the simple approach of external calibration in water after a standard two-phase acid extraction of cultured cells. This assay, allowing direct GroPIns quantitation down to 5 ng/ml, thus represents a more advantageous alternative to the [^{3}H]-*myo*-inositol labelling that has been commonly used for this compound as a detection and quantitation technique. Comparison of data obtained for extracts of selected cell lines with both the external calibration and standard additions methods has demonstrated external calibration to be a feasible and accurate approach to GroPIns quantitation in cell extracts, with the advantage that it is both simpler and less time-consuming than the standard additions method.

Furthermore, monitoring of further MRM transitions specific for the phosphorylated forms of GroPIns provides the potential for the concurrent detection and quantitation of these compounds, which are the direct deacylation products of the full range of the naturally occurring membrane phosphoinositides, dependent upon their own ESI-MS sensitivities and their levels in similar cell extracts. The importance of this further application can be seen in the wide roles that these membrane phosphoinositides have in the mediation of cell growth and proliferation, apoptosis, cytoskeletal changes, insulin action and vesicle trafficking [28].

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