Optimization of monosaccharide determination using anthranilic acid and 1-phenyl-3-methyl-5-pyrazolone for gastropod analysis

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ABSTRACT

The protein-linked glycomes and, thereby, the range of individual monosaccharides of invertebrates differ from those of mammals due to a number of special modifications; therefore, it is necessary to adapt methods for monosaccharide analysis in order to cover these. We optimized the labeling procedure for anthranilic acid (AA) and 1-phenyl-3-methyl-5-pyrazolone (PMP) and the subsequent separation of the labeled monosaccharides on high-performance liquid chromatography (HPLC), with the result that we were able to identify 26 different monosaccharides. The detection limit for anthranilic acid derivatives obtained was 65 fmol, and a reliable quantification of samples was possible up to 200 nmol under the tested conditions. PMP derivatives showed a significantly higher detection limit but allow quantification of larger sample amounts. Applying these methods on snails, their impressive set of monosaccharide constituents, including methylated sugars, was shown.

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Glycans play important roles in many recognition processes such as fertilization and development, allergies, pathological events, and cell death. Therefore, their detailed analysis is an important issue to get a closer view of these processes. As a first step, usually monosaccharide constituent analysis is performed. Several methods have been established employing liquid chromatography, gas chromatography, or capillary electrophoresis. However, each of these methods has its limitations in terms of selectivity, separation, detection, sensitivity, and/or effort for sample preparation. To detect monosaccharides following liquid chromatography or capillary electrophoresis, it is necessary to label them by a fluorescent or ultraviolet (UV) 1 tag. 2-Aminobenzoic acid, also known as anthranilic acid (AA), and 1-phenyl-3-methyl-5-pyrazolone (PMP) are the most commonly used tags. Both are highly reproducible and accurate. The reaction scheme and detailed chemistry for the derivatization with AA were well described in Refs. [1,2], and the derivatization with PMP was described in Ref. [3]. So far, most of the studies have concentrated on the analysis of GalN, GlcN, Man, Gal, Glc, and Fuc due to their frequent occurrence in mammalian glycans. If plant material is analyzed, Xyl and Ara are also included. However, increasingly the focus is on the glycosylation capacities of microorganisms, parasites, or invertebrates that may contain uncommon monosaccharide constituents. Snails, for example, show methylated mannoses and galactoses [4]. Therefore, we expanded the spectrum of the usual monosaccharides for high-performance liquid chromatography (HPLC)-based analyses by methylated sugars and other rare sugars to examine up to 26 different monosaccharides, thereby aiding studies on uncommon glycan structures. Furthermore, we improved and optimized these well-established methods in terms of detection limit, minimal sample amount, and separation capacity to make them applicable for the analysis of gastropod glycoconjugates.

Materials and methods

Materials

Cepaea hortensis, Planorbarius corneus, Arion lusitanicus, Helix pomatia, and Arianta arbustorum were collected by the authors under the supervision of Manfred Pintar (Department of Integrative Biology and Biodiversity Research, Institute of Zoology, University of Natural Resources and Life Sciences, Vienna, Austria) in areas close to Vienna. Achatina fulica and Biomphalaria glabrata were bred in the laboratory at 25 °C. All animals were frozen at –80 °C immediately after collection. 2- and 4-O-Methylated Gal were provided by Paul Kosma (Department of Chemistry, University of Natural Resources and Life Sciences, Vienna, Austria), and 3-O-methylated GlcN and 3-O-methylated GlcNAc were provided by Athanasios Giannis (Institute of Organic Chemistry, University of Leipzig, Leipzig, Germany). All chemicals purchased were of the highest quality available from Sigma or Fluka.
Derivatization with AA and separation on reversed phase HPLC

The derivatization was modified and downscaled from Ref. [5] to optimize the ratio between reagent and monosaccharide peaks, especially in the case of samples containing low amounts of carbohydrate. The whole assay was scaled down to 1/10 of the original protocol, whereas the amount of reagent could be reduced to 1/100 in total. Dry monosaccharides (180 nmol each for standard procedure) were dissolved in 5 μl of sodium acetate trihydrate solution (80 mg/ml) by mixing vigorously with a vortex mixer. Then 10 μl of AA reagent solution (3 mg of AA and 30 mg of sodium cyanoborohydride in 1 ml of 2% [w/v] boric acid in methanol) was added and incubated for 60 min at 80 °C. Samples were diluted 1:500 prior to HPLC analysis. Analysis was carried out on an Agilent 1200 LC (liquid chromatography) system at a constant flow rate of 1 ml/min with fluorometric detection at 360 nm excitation and 425 nm emission using a reversed phase C18 column (ODS Hypersil, 5 μm, 250 × 4 mm, Thermo Scientific, part no. 30105-254030). Solvent A was composed of 1.0% tetrahydrofuran, 0.5% acetonitrile, and 0.8% acetonitrile in 0.1 M ammonium acetate buffer (pH 5.5), and water. Solvent B was a mixture of equal amounts of solvent A and acetonitrile. After injection of 5 μl of sample, a linear gradient of 5–25% B was applied over 17 min. For the next 6 min, another gradient up to 100% B was applied. To wash and reequilibrate the column, the run was continued by an isocratic elution with 100% B for 6 min, followed by a gradient of 100 to 5% B over 1 min. Finally, 5 min of initial conditions was applied. For quantitative analysis, samples in the range from 0.5 to 350 nmol of monosaccharide (Gal, Man, GlcN, GalN, Xyl, and Fuc) were derivatized according to the standard protocol. The limits of detection (corresponding to a signal-to-noise ratio of 3) and quantitation (corresponding to a signal-to-noise ratio of 10) were calculated according to the guidelines of the European Pharmacopoeia [6]. Background noise was determined as 6 times the standard deviation of the linear regression of the drift in a chromatogram obtained after application of a blank by the Agilent ChemStation for LC 3D Systems software (version B.04.01 SP1). Ranges for background noise calculation were set as the distance of 20 times the width at half-height of the corresponding reference peak distributed equally around respective retention times. All experiments were carried out at least in triplicate.

Derivatization with PMP and separation on reversed phase HPLC

The derivatization was modified from Ref. [7]. Dry monosaccharides (180 nmol each for standard procedure) were dissolved in 25 μl of a solution of 0.5 M PMP in methanol. After adding 15 μl of 0.5 M NaOH and 10 μl of water, the mixture was incubated for 120 min at 70 °C. For neutralization, 20 μl of 0.5 M HCl was added. The mixture was extracted 5 times by the addition of 500 μl of organic solvent (for standards, diethyl ether was used and dichloromethane and butylether were also tried), followed by vigorous mixing and centrifugation at 3000g for 1 min. The organic layer was removed carefully. In contrast to the previous publication [7], the final aqueous layer was evaporated to dryness and then redissolved in 1 ml of water, yielding a standardized final volume. HPLC analysis was carried out on a reversed phase C18 column (ODS Hypersil, 5 μm, 250 × 4 mm) on an Agilent 1200 LC system at a constant flow rate of 1.5 ml/min. Solvent A was composed of 8% acetonitrile in 0.1 M ammonium acetate buffer (pH 5.5), and solvent B was composed of 30% acetonitrile in 0.1 M ammonium acetate buffer (pH 5.5). After injection of 5 μl of sample, a linear gradient of 45–50% B was applied over 10 min. Next, a gradient of 50–85% B was applied over 16 min. Finally, 5 min of initial conditions was applied to reequilibrate the column for further analyses. Detection was carried out at 245 nm. For quantitative analysis, samples in the range from 0.5 to 350 nmol of monosaccharide (Gal, Man, GlcN, GalN, Xyl, and Fuc) were derivatized according to the standard protocol. Limits of detection and quantification were determined similar to the AA method. All experiments were carried out at least in triplicate.

Preparation of proteins from snail origin

Snails were dissected as described previously [8], and proteins were purified according to Ref. [9] with minor modifications. Wet tissue (10 g) was homogenized in 50 ml of Chaps-based lysis buffer (0.5% [w/v] Chaps, 150 mM NaCl, 20 mM Tris/HCl, 2.5 mM sodium pyrophosphate, 1 mM ethyleneglycol-bis(2-aminoethylether)-N,N,N,N-tetraacetic acid, and 1 mM ethylenediaminetetraacetic acid [EDTA], pH 7.5) and incubated for 60 min at 4 °C. Insoluble compounds were removed by a centrifugation step of 30 min at 10,000g. Next, 2.5 ml of 100 mM diithiothreitol was added to 50 ml of supernatant and incubated for 45 min at 56 °C. After that, 2.5 ml of 55 mM iodoacetamide was added and incubated for 30 min at room temperature in the dark. The samples were dialyzed against water overnight and lyophilized.

Hydrolysis and analysis of snail-derived monosaccharides

An aliquot of the purified (glyco)proteins containing approximately 50 μg of carbohydrate was dissolved in 300 μl of 4 M trifluoroacetic acid and hydrolyzed for 120 min at 115 °C. The samples were dried under reduced pressure prior to further 3-fold reevaporation from 500 ml of 30% (v/v) methanol. The dried samples were subject to the derivatization and separation protocols described above for AA and PMP.

Results and discussion

To be able to identify a broad range of monosaccharides by their retention times, two independent derivatization reagents that cause different elution behaviors of the labeled monosaccharides on reversed phase HPLC were chosen. The combined evaluation of the data of both methods enables a precise identification of each monosaccharide.

Derivatization with AA

Labeling of monosaccharides with the fluorescent marker AA is known for its high sensitivity and the easy and fast derivatization chemistry [1,2]. An advantage of this method is that no re-N-acetylation is necessary after hydrolysis. Method validation for five sugars has been done previously [10]. Nevertheless, HPLC analysis of samples with low carbohydrate content sometimes causes problems due to the large peak of excess reagent and the relatively small monosaccharide peaks. Attempts to increase the signals of the labeled sugars in contrast to the reagent peak by optimizing the wavelengths of excitation and the emission did not succeed. However, following further experiments regarding the derivatization procedure, we were able to scale down the method of Anumula [11] in order to fit the requirements for gastropod monosaccharide analysis. Using the original protocol, almost similar peak heights for the reagent peak were obtained within a range of 0.5–5 μmol of sugar, whereas the sugar peak increased proportionally (data not shown). Because our usual amount of monosaccharides does not exceed 0.5 μmol, we reduced the total reagent volume by a factor of 10, dissolving the sample in 5 μl of sodium acetate solution and adding only 10 μl of reaction solution. In addition, we reduced the amount of AA from 30 to 3 mg per milliliter of AA reaction solution while keeping the amount of sodium cyanoborohydride constant. This improved the ratio of the sugar and
AA peaks and kept the concentration of the reaction mixture high enough to ensure proper derivatization of small amounts of sugar (Fig. 1). Under our conditions, we did not observe any epimerization from GlcN to ManN during derivatization as reported previously [10]. The limits of detection (65 fmol) and quantitation (220 fmol) were calculated according to the guidelines of the European Pharmacopoeia [6]. However, we recommend a working limit of 350 fmol, which is in accordance with previous publications [12].

Separation of 26 AA-labeled monosaccharides

A great advantage of AA-labeled monosaccharides is the different elution behavior of amino sugars as opposed to the other monosaccharides on reversed phase HPLC. They are clearly distinguishable by their elution before (amino sugars) or after (all other monosaccharides analyzed so far) the reagent peak. Methylated monosaccharides elute significantly later than all other sugars. The separation capacity of the chosen gradient is optimal. Even with the high number of sugars, we were able to attain a run time of 35 min rather than 2 h as published previously [10]. Five pairs of sugars that cannot be distinguished by this method were found: allose/Man, Ara/altrose, Rha/Fuc, 4-O-Me-Glc, and 3-O-Me-GlcN/2-O-Me-Gal (where Me represents methyl). Even changes in the solvents or the gradient did not improve their separation. However, these pairs are effectively separated in the PMP method; therefore, the identification of these sugars is unambiguous. For a standard run of AA-labeled sugars, see Fig. 2. The retention times are listed in Table 1.

Quantification of sugars labeled with AA

Calibration curves have been established for GlcN, GaN, Man, Gal, Xyl, and Fuc by derivatizing different amounts (from 0.1 to 100 µg, which equates to an approximate range of 0.5–500 nmol depending on the particular sugar) of the monosaccharides under standard conditions. The curves obtained show a linear range for the derivatization of amino sugars from 0.5 to 100 nmol and for the other sugars from 0.5 to 200 nmol (Fig. 3A). Whereas the peak areas of Gal and Man showed comparable values at certain amounts of derivatized sugar, Xyl and Fuc showed slightly higher response factors and GlcN and GaN showed significantly lower response factors. Because we also found that the derivatization efficiency of 2-O-methylated galactose is significantly lower than that of 4-O-methylated galactose, we presume that additional groups at the C2 position of the sugar ring negatively interfere with the derivatization at the reducing end.

Derivatization with PMP

Labeling with the UV tag PMP is also a relatively simple process, and previous optimizations of this method have been described by Honda and coworkers [3] and Fu and O’Neill [7]. Similar to the AA method, it also does not require re-N-acetylation after hydrolysis. However, in contrast to AA labeling, the sample needs to undergo further purification after the PMP derivatization because the excess reagent must be extracted by organic solvent. Diethyl ether, dichloromethane, and butylether were tried. For standard preparation, diethyl ether was chosen because in our experiments it gave the best ratio between sugar and reagent peak. Previous publications have
suggested performing the extraction two or three times, which reduces the reagent peak drastically. We found that five extraction steps are necessary to remove excess reagent completely. Limits of detection and quantitation were determined as described above. Due to an increased baseline drift toward the end of the run, signal-to-noise ratios vary significantly between early and late eluting sugars. Therefore, limits of detection and quantitation vary between 0.2 pmol (detection)/0.7 pmol (quantitation) for the early eluting sugars. Thus, detection and quantitation were determined as described above. Due to an increased baseline drift toward the end of the run, signal-to-noise ratios vary significantly between early and late eluting sugars. Therefore, limits of detection and quantitation vary between 0.2 pmol (detection)/0.7 pmol (quantitation) for the early eluting sugars. Thus, detection and quantitation were determined as described above.

**Separation of 26 PMP-labeled monosaccharides**

In contrast to the AA method, retention times on HPLC after PMP labeling allow no easy prediction of the nature of a sugar. Amino sugars, as well as methylated sugars, are scattered throughout the whole chromatogram. All sugar peaks elute after the reagent peak starting with Man. Again some monosaccharides cannot be separated, but because they form two pairs (Ara/Xyl and Rib/Rha) that are clearly distinguishable after AA labeling, their identification proves to be unambiguous. For a standard run, see Fig. 4. The retention times of all sugars are listed in Table 1.

**Quantification of sugars labeled with PMP**

Calibration curves have been established for GlcN, GalN, Man, Gal, Xyl, and Fuc by derivatizing different amounts (from 0.1 to 100 μg, which equates to an approximate range of 0.5–500 nmol depending on the particular sugar) of the monosaccharides under standard conditions. For Gal, Man, Fuc, and Xyl, the method showed a linear range throughout our test assay. For amino sugars, the linear range was significantly smaller, just up to approximately 300 nmol. In contrast to the AA derivatization method, where the amount of AA used limits the maximum amount of sugar that can be derivatized, PMP is used in high excess and, therefore, needs to be removed before HPLC analysis. Similar to AA derivatization, peak areas for amino sugars were significantly smaller than those for other sugars when the same amount of monosaccharide was derivatized. The other sugars—Man, Gal, Xyl, and Fuc—show comparable results, but it should be noted that late eluting sugars show peak broadening, and therefore significant lower peak heights, for comparable peak area values. For the linear regions of the calibration curves, see Fig. 3B. Our data for Gal, Glc, Xyl, and Fuc correlate well with those published previously [7], but our curves for the amino sugars were suboptimal. This is due to the different methodological approaches. In the previous publication, the curves represent dilution series of one derivatized sample, where 10 nmol of each sugar was derivatized and varying amounts of the single final product were analyzed by HPLC. We performed separate derivatizations of different amounts of each sugar to obtain the points of the calibration curves. Therefore, our calibration curves include all derivatization and purification effects using these different concentrations. We believe that this methodical strategy correlates better with a broader range of sample types.

**Monosaccharide analysis of snail glycans**

Monosaccharide analysis using the two labeling techniques described above was carried out with seven species of snails: two water snails (B. glabrata and P. corneus), one land living slug (A. lusitanicus), and four land living snails carrying shells (A. arbustorum, A. fulica, C. hortensis, and H. pomatia). All of them contain an impressive set of monosaccharides. Besides those occurring frequently in nearly all higher organisms, snails also contain methylated variants of hexoses, typically 3-O-methylated Man, 3-O-methylated Gal, and 4-O-methylated Gal. Those have been detected and identified by gas chromatography/mass spectrometry in detail for some species recently [4].

For the snail tissues, the combination of these two methods allows a precise identification of all major peaks. Of course, the analysis of many compounds in quite short runs may result in rather small differences in elution times. Here we used the second system to resolve pairs whose identification was ambiguous. For example, AA-labeled Rha and Fuc elute at 17.55 and 17.56 min, respectively, and cannot be distinguished; however, when labeled with PMP, they show rather divergent elution times (11.11 min for Rha and 21.29 min for Fuc), making it easy to prove that the snails contain Fuc but do not contain any Rha. Some of our snail tissues contain...
remarkable amounts of ribose and deoxyribose. This may be due to the developmental stage of the cells and the RNA and DNA content, respectively. Also in the monosaccharide patterns of microorganisms during the logarithmic reproduction stage, the amount of ribose and deoxyribose is strongly increased (data not shown).

Methylated sugars labeled with AA elute in the last part of the chromatogram, whereas those labeled with PMP are distributed over the whole run. A problem in the analysis of methylated sugars was the poor availability of standard monosaccharides. Whereas a 4-O-methylated Gal standard could be obtained, there was no source for the 3-O-methylated Man or 3-O-methylated Gal available. However, in comparing the previous quantification data from the gas chromatography analysis with the HPLC patterns, we were able to identify the appropriate signals. Most of our snail patterns contained two obvious peaks that did not comigrate with any of our other 24 standard monosaccharides and that appeared close to other methylated sugars in AA analysis. From gas chromatography/mass spectrometry analysis, we knew that there should be significant amounts of 3-O-methylated Gal and 3-O-methylated Man in most of the snails as well as some quantitative differences in some others [4]. Both B. glabrata and A. fulica show a significant amount of 3-O-methylated Man. 3-O-Methylated Gal is clearly present in B. glabrata, whereas it is just a minor compound in A. fulica. This indicated that the last peak of the AA pattern (21.85 min) is 3-O-methylated Man, whereas the previous one (21.59 min) is 3-O-methylated Gal. In the PMP pattern, 3-O-methylated Man elutes at 14.76 min and 3-O-methylated Gal elutes at 20.06 min. Those findings were confirmed by the analysis of H. pomatia, which shows a clearly 3-O-methylated Gal peak compared with the 3-O-methylated Man signal (Figs. 5 and 6). All of these data correlate with the amounts obtained by gas chromatography/mass spectrometry analysis [4]. Therefore, we are confident that these two sugars could be included in our table of retention times.

The monosaccharide patterns of A. lusitanicus, A. fulica, and H. pomatia shown in Figs. 5 and 6 are typical of the results for snail material. All of the commonly found sugars (GaN, GlcN, Man, Glc, Gal, and Fuc) are clearly present; the amount of Xyl and methylated hexoses (3-O-Me-Man, 3-O-Me-Gal, and 4-O-Me-Gal) varies with the species. No special similarities were found to distinguish between land- and water-living animals or between those with and without shells.

With two different labeling techniques (using AA and PMP), we could separate 26 different monosaccharides on reverse phase HPLC, including several methylated sugars that were not examined previously by these systems. The short run times (<40 min) also present a significant optimization and so are applicable for routine use. Because retention times may shift slightly by using a column from another supplier or batch and also with changes in the temperature, standard runs before and after the sample runs are essential. Because the order of the peaks remains constant, the system is a valuable analytical tool for the identification of monosaccharides. Those monosaccharides coeluting in one method can be clearly separated with the other method. Each sugar examined can be completely resolved from every other sugar by at least one of the two methods. The combination of these two methods provides a reliable detection system for identification as well as quantification. Whereas fluorescent detection of the AA tag facilitates analysis of very small sample amounts after a fast single step derivatization, the more time-consuming PMP derivatization technique is a valuable addition for the quantification of larger sample amounts.

In optimizing both derivatization protocols, we were able to detect 65 fmol of sugar using the AA method and to quantify sample amounts up to 300 nmol of sugar using the PMP method. Applying this system to gastropod monosaccharide analysis confirmed the broad spectrum of monosaccharides in snails as well as the presence of methylation as a feature of glycoconjugates of all snail species investigated.

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