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Research paper

Development of antibodies for determination of alkylresorcinol metabolites in human urine and elucidation of ELISA cross-reactivity

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ABSTRACT

Alkylresorcinols (ARs) are amphiphilic phenolic lipids and their two main metabolites, 3-(3,5-dihydroxyphenyl)-propanoic acid (DHPPA) and 3,5-dihydroxybenzoic acid (DHBA), can be used as biomarkers of whole grain wheat and rye intake. The aim of this study was to develop antibodies against DHBA and DHPPA for use in ELISA analysis. Good calibration curves were obtained for ELISA using alkaline phosphatase (AP) conjugates. The highest sensitivity for DHPPA was found using a reagent combination of anti-DHPPA–BSA and DHPAA–AP in a direct ELISA (IC_{50} = 1.5 μmol/L), and for DHBA using a reagent combination of anti-DHBA–OV and DHBA–AP (IC_{50} = 1.3 μmol/L). Calibration was conducted in the linear range (0.3–27.4 μmol/L), with limit of detection (LOD) 0.1 μmol/L. Intra and inter CVs was in the range of 0.7–7.2% and 5.1–11.5%, respectively, for DHPPA and 1.3–9.4% and 3.5–20%, respectively, for DHBA. Mean recovery was 104% for DHPPA and 102% for DHBA. The ELISA method developed was then used for analysis of 120 urine samples from free-living men and women that had previously been analysed by gas chromatography–mass spectrometry (GC–MS). ELISA produced several-fold higher values than GC–MS. Application of high-resolution Orbitrap mass spectrometry (HR Orbitrap MS) allowed several compounds, including novel putative AR metabolites, to be identified, synthesised and confirmed as compounds with high ELISA cross-reactivity.

Abbreviations: ADHBA, 4-Amino-3,5-dihydroxybenzoic acid; AP, alkaline phosphatase; AR, alkylresorcinol; BSA, bovine serum albumin; CR, cross-reactivity; CV, coefficient of variation; DCC, dicyclohexylcarbodiimide; DHBA–glycine, 2-(3,5-Dihydroxybenzamido)acetic acid; DHBA, 1,3-Dihydroxy-benzoic acid; DHCA, 3,5-Dihydroxycinnamic acid; DHCA-amide, 3,5-Dihydroxycinnamic acid amide; DHPAA, 3,5-Dihydroxyphenyl acetic acid; DHPPA, 3-(3,5-Dihydroxyphenyl)-propanoic acid; DHPPTA, 5-(3,5-Dihydroxyphenyl)pentanoic acid; DMF, N,N-dimethylformamide; ELISA, enzyme-linked immunosorbent assay; ESI, electrospray ionisation; FCA, Freund's complete adjuvant; FIA, Freund's incomplete adjuvant; GC–MS, gas chromatography–mass spectrometry; HCD, high energy collision-induced dissociation; HESI II, heated electrospray ion source; HPLC, high performance liquid chromatography; HR Orbitrap MS, high-resolution Orbitrap mass spectrometry; HRP, horseradish peroxidase; IS, internal standard; LOD, limit of detection; LOQ, limit of quantification; MS, mass spectrometry; NHS, N-hydroxysuccinimide; OV, ovalbumin; PBS, phosphate-buffered saline; SwAR, swine immunoglobulin against rabbit immunoglobulin labelled with HRP; TG, porcine thyroglobulin; TMB, 3,3′,5,5′-Tetramethylbenzidine.

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1. Introduction

Alkylresorcinols (ARs), amphiphilic 1,3-dihydroxy-5-alkyl benzenes, have been proposed as specific dietary biomarkers of whole grain wheat and rye intake (Chen et al., 2004; Ross et al., 2004; Landberg et al., 2008, 2009b). AR homologues (17–25 carbon in the alkyl tail) are extensively metabolised in the liver and form the two main metabolites 1,3-dihydroxybenzoic acid (DHBA) and 3-(3,5-dihydroxyphenyl)-1-propanoic acid (DHPPA). DHBA and DHPPA can be detected and quantified as a mixture of aglycones and glucuronide and sulphate conjugates in urine (Ross et al., 2004). In a recent study, 5-(3,5-dihydroxyphenyl) pentanoic acid (DHPPTA) and 2-(3,5-dihydroxybenzamido)acetic acid (DHBA–glycine) were identified as metabolites from ARs, but they are present at low concentrations in urine (Zhu et al., 2013). Moreover, 3,5-dihydroxycinnamic acid sulphate has been identified in human urine and is suggested to be an AR metabolite (Bondia-Pons et al., 2013). Under some conditions, AR metabolites such as DHBA and DHPPA may also be suitable as dietary biomarkers for the intake of whole grain wheat and rye intake, particularly in 24-h urine collections (Landberg et al., 2009a; Marklund et al., 2010).

For applications of biomarkers in large-scale epidemiological studies, large numbers of samples typically need to be analysed. Available methods for quantification of AR metabolites in urine include gas chromatography–mass spectrometry (GC–MS) and high-performance liquid chromatography (HPLC) coupled to a coulometric electrode array detector (Ross et al., 2001; Koskela et al., 2007, 2008; Marklund et al., 2011). These analytical methods are accurate, highly specific and sensitive, but they are also relatively expensive, time-consuming and not ideally suited for analysing large amounts of samples in a short time.

Immunoassay could be a suitable method for analysing large series of samples, e.g. blood and urine samples for epidemiological studies, due to the simple, quick and relatively inexpensive assay procedure (Cespedes et al., 2006; Franek et al., 2006; Vass et al., 2008; Cernoch et al., 2011). The specificity and affinity of antibodies incorporated into the test format determine the analytical characteristics of the detection system. Therefore, optimal haptens need to be designed in order to obtain analyte-specific antibodies. It is common immunochemical experience that the position of spacers in the hapten structure plays a key role in antibody specificity (Eyer and Franek, 2012).

In this paper, we report production and characterisation of rabbit antibodies against DHBA and DHPPA and their utilisation for the development of competitive ELISA systems. The optimised ELISA method was used for determination of DHBA and DHPPA in 120 urine samples taken from free-living men and women and previously quantified by GC–MS. A high-resolution Orbitrap mass spectrometry (HR Orbitrap MS) approach was used to identify potential cross-reacting compounds in the ELISA.

2. Experimental

2.1. Chemicals and reagents

DHBA, DHPPA, DHPAA, DHPPTA and 3,5-DHBA–glycine were purchased from ReseaChem (Burgdorf, Switzerland). ADHBA (4-amino-3,5-dihydroxybenzoic acid), DHCA (3,5-dihydroxycinnamic acid) and DHCA amide (3,5-dihydroxycinnamic acid amide) were synthesised by T. Vontor (Hradec Kralove, Czech Republic). Bovine serum albumin (BSA), ovalbumin (OV), porcine thyroglobulin (TG), casein, Freund's complete adjuvant (FCA), Freund's incomplete adjuvant (FIA), ethyl acetate, glycerol, Sephadex G-25, peroxidase type II from horseradish (HRP), polyethylene-sorbitanmonolaurate (Tween-20), N-hydroxysuccinimide (NHS), dicyclohexylcarbodiimide (DCC), N,N-dimethylformamide (DMF), diethanolamine, p-nitrophenyl phosphate and 3,3′,5,5′-tetramethylbenzidine (TMB) were obtained from Sigma-Aldrich Chemie (Steinheim, Germany). Alkaline phosphatase (AP) was obtained from New England BioLab (United Kingdom). Swine immunoglobulin against rabbit immunoglobulin labelled with HRP (SwAR) was purchased from Servapharma (Prague, Czech Republic). Methanol, diethylether, formic acid and ethyl acetate of high performance liquid chromatography (HPLC) grade were purchased from Sigma-Aldrich (Prague, Czech Republic). Deionised water was produced with the Nanopure system from GORO (Prague, Czech Republic). The internal standard (IS) syringic acid was purchased from Sigma-Aldrich (Prague, Czech Republic).

2.2. DHCA and DHCA-amide syntheses

Two putative AR metabolites, DHCA and DHCA-amides, were synthesised in-house as described below.

2.2.1. Synthesis of DHCA

In brief, 2.5 g (0.024 M) malonic acid was dissolved in 20 mL dry pyridine and after 5 min 2.8 g 3,5-dihydroxybenzaldehyde (0.02 M) and 0.1 mL piperidine (catalyst) were added. The solution was heated until carbon dioxide developed (approx. 6 h). After cooling, the solution was poured into cold water containing hydrochloric acid. The raw product was aspirated off, washed with water and after drying it was purified by means of column chromatography (silica gel, ethyl acetate-methanol 4/1). The presence of DHCA in the final product was confirmed by NMR analysis. The product contained 98% DHCA.

2.2.2. Synthesis of DHCA-amide

In brief, 3.2 g (0.024 M) monoethyl malonate was dissolved in 20 mL dry pyridine and after 5 min 2.8 g (0.02 M) 3,5-dihydroxybenzaldehyde and 0.1 mL piperidine were added. The solution was heated until carbon dioxide developed (approx. 6 h). After cooling, the solution was poured into cold water containing hydrochloric acid. The ethyl 1,3-dihydroxycinnamate obtained was aspirated off, washed with water and, after drying, purified by column chromatography (silica gel, $CHCl_3$–methanol 4/1). Then 2.1 g (0.01 M) ethyl 3,5-dihydroxycinnamate was mixed with 30 mL concentrated ammonia for 24 h. The reaction mixture was acidified (HCl) to reach pH 2. The solid portion was filtered off, dried and purified by column chromatography (silica gel, ethyl acetate). The presence of the DHCA-amide in the final product was confirmed by NMR analysis. The product contained 78% DHCA-amide, 17% DHCA and 5% other unidentified compounds.