

Evaluation of an immunoaffinity extraction column for enrichment of adducts between human serum albumin and hexahydrophthalic anhydride in plasma

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ABSTRACT: An immunoaffinity extraction (IAE) column was prepared for extraction of adducts between human serum albumin (HSA) and hexahydrophthalic anhydride (HHPA). HHPA is a strong sensitizer inducing immunoglobulin E antibodies *in vivo*. Polyclonal antibodies from a rabbit immunized with keyhole limpet hemocyanin–HHPA conjugate were purified using a Protein A Sepharose gel. To obtain antibodies with optimal affinity towards HHPA–protein adducts, HHPA-specific antibodies were selected using an *N*-hydroxysuccinimide–Sepharose column coupled with albumin–HHPA conjugate. Antibodies eluted from this column at pH 2.2 were selected to prepare the IAE column. The column was evaluated using 2 mL plasma spiked with HSA–HHPA conjugate. The column was eluted with glycine buffer at pH 2.0. The conjugates in the eluate were hydrolyzed to the corresponding HHP acid and quantified by mass spectrometry. The average recovery of HHPA adducts in 11 experiments was 68% with a coefficient of variation (CV) of 7%. The column's capacity to bind protein–HHPA adducts was found to be linear in the range of 0.15–1.2 nmol conjugate. The evaluation showed that the IAE column had adequate affinity towards the HHPA adducts and that the adducts could be extracted with good recovery and precision from a large volume of plasma. Copyright © 2007 John Wiley & Sons, Ltd.

KEYWORDS: immunoaffinity extraction; mass spectrometry; protein adducts

INTRODUCTION

Analysis of low levels of proteins or peptides in complex matrices such as plasma or serum is difficult. Although enzyme-linked immunosorbent assays (ELISA) can be used for targeted protein analysis, there are concerns of selectivity. However, it is also difficult to use specific methods like mass spectrometry without prior enrichment of the samples (Ackermann and Berna, 2007). To improve the detection limit of low abundance analytes it would be of high value to isolate and enrich these by selective extraction, thus lowering the background and

making detection feasible. Such enrichment of proteins may be accomplished through immunoaffinity extraction (IAE) on the basis of specific antibody–antigen interactions. Analytes such as proteins (Hage, 1999; Ma and Sayeski, 2004), modified peptides (Boysen *et al.*, 2004; Mano *et al.*, 2006) or small molecules such as organic pollutants, pesticides and drugs (Delaunay *et al.*, 2000) have been subjected to immunoaffinity extraction.

The complex formed when a chemical binds to a larger carrier molecule is called an adduct (Törnqvist *et al.*, 2002). In the context of this paper, we refer to an adduct as the moiety of the reactive compound covalently bound to the peptide sequence and the whole carrier protein with one or several adducts is referred to as a conjugate. Quantification of protein adducts can be used as a long-term biomarker of exposure.

Organic acid anhydrides (OAA) may induce work-related symptoms of the upper and lower airways (Moller *et al.*, 1985; Venables, 1989). Some of the symptoms arise from type-1 allergy mediated by IgE antibodies as shown by Nielsen *et al.* (1994). HHPA has been shown to be a particularly strong sensitizer (Welinder *et al.*, 1994; Nielsen *et al.*, 2001). Proteins have been shown to form adducts with HHPA *in vivo* (Johannesson *et al.*, 2001). Such bindings may induce production of specific IgE or affect physiological

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Abbreviations used: ESI, electrospray ionization; GC/MS, gas chromatography/mass spectrometry; HHPA, hexahydrophthalic anhydride; HHP acid, hexahydrophthalic acid; HSA, human serum albumin; IAE, immunoaffinity extraction; Ig, immunoglobulin; KLH, keyhole limpet hemocyanin; LC/MS/MS, liquid chromatography/tandem mass spectrometry; NHS, *N*-hydroxysuccinimide; OAA, organic acid anhydride; PBS, phosphate-buffered saline; RSA, rabbit serum albumin; SPE, solid-phase extraction.

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mechanisms of the proteins and thus be important in the mechanisms behind allergy (Nielsen *et al.*, 1994). The protein adducts of HHPA in occupationally exposed workers are in the low pmol/mL plasma levels (Rosqvist *et al.*, 2001).

In this study HHPA and HHPA adducts were used as model compounds for the evaluation of an IAE column. There are several well-validated analytical methods described for quantitative analysis of the protein adducts. After an acid hydrolysis of the protein adducts, the corresponding HHP acid is released. The HHP acid can then be analysed using liquid chromatography tandem MS (LC/MS/MS; Kristiansson *et al.*, 2002) or after derivatization using gas chromatography mass spectrometry (GC/MS; Lindh and Jönsson, 1998).

The aim of this study was to develop and evaluate an immunoaffinity extraction column for enrichment of protein adducts from large volumes of plasma using HSA-HHPA conjugates as model compounds.

EXPERIMENTAL

Chemicals and reagents. *Caution:* HHPA is a hazardous chemical and must be handled using proper safety measures. HHPA was from Acros Organics (Geel, Belgium) and [$^2\text{H}_6$]-HHP acid was obtained from Synthelec (Lund Sweden). KLH was from ICN Biomedicals (Costa Mesa, CA, USA), Protein A Sepharose and *N*-hydroxysuccinimide (NHS)-activated HiTrap Sepharose were from Amersham Pharmacia Biotech (Uppsala, Sweden) and dimethyl pimelimidate-2 HCl (DMP) was from Pierce (Rockford, IL, USA). HSA, rabbit serum albumin (RSA) fraction V and pentafluorobenzyl bromide (PFBBBr) were from Sigma Chemicals (St Louis, MO, USA). All other chemicals and solvents were of analytical grade from commercial sources. Standards of HHP acid were prepared by hydrolyzing HHPA in water and then further diluting with water. Normal human plasma samples were obtained from a blood bank. The dialysis was performed using Spectra/Por molecular porous membrane tubing with a cutoff at 12–14 kDa, from Spectrum (Gardena, CA, USA).

Preparation and purification of KLH-HHPA antiserum. Following the protocol by Johannesson *et al.* (2004) conjugates between KLH and HHPA were prepared at pH 8.1 and then dialyzed. KLH-HHPA antiserum was produced by immunizing a rabbit with repeated injections of these KLH-HHPA conjugates at Antibody AB (Lund, Sweden). The IgG pool was isolated from the antiserum by purification using a Protein A Sepharose gel according to manufacturer's instructions.

In vitro synthesis of RSA-HHPA conjugate. The RSA-HHPA conjugate was synthesized according to Kristiansson *et al.* (2003). Briefly, HHPA was dissolved in acetonitrile and added to a solution of RSA dissolved in PBS. Conjugates with two different molar ratios of added HHPA were used, RSA-HHPA 1:5 and 1:50, in order to get a mixture of hapten densities. The solutions were incubated at room temperature for 2 h and were then transferred to dialysis membranes. The

conjugates were dialyzed in PBS with 2 mM ethylenediamine-tetraacetic acid (EDTA) for 36 h and then in 0.2 M NaHCO_3 (pH 8.1), 0.5 M NaCl for 24 h.

Immobilization of RSA-HHPA conjugates on NHS-activated HiTrap Sepharose and selection of HHPA-specific IgG. One milliliter of RSA-HHPA conjugate (5 mg/mL) with molar ratios of 1:5 and 1:50 were covalently bound to the NHS-activated HiTrap Sepharose (1 mL column) according to the manufacturer's instructions. The column was equilibrated in 0.5 M NaCl in 50 mM Tris-HCl (pH 7.4).

The IgG pool previously purified on the Protein A Sepharose gel was dialyzed in 50 mM Tris-HCl (pH 7.4), 0.5 M NaCl. Thereafter, the IgG pool was recirculated for 2 h at a flow rate of 0.8 mL/min on the RSA-HHPA conjugate-NHS-activated HiTrap Sepharose.

The column was washed with 0.5 M NaCl in 50 mM Tris-HCl (pH 7.4) buffer. The IgG pools were eluted in three steps using 0.5 M NaCl in 0.1 M glycine buffer with pH 4.5, 3.6 and 2.2. The obtained fractions were visualized using Coomassie staining on an agarose gel. The IgG antibodies eluted with pH 3.6 were selected for the method and dialyzed in 0.5 M NaCl in 50 mM Tris-HCl (pH 7.4).

Preparation of the IAE column. The selected antibodies were immobilized on the Protein A Sepharose support, using the method of Friesen *et al.* (1991), with some modifications. The suspension was rotated overnight, at 4°C, on a roller mixer. UV absorbance at $\lambda = 280$ nm was measured in the IgG solution before and after coupling to the Protein A Sepharose. The gel was recovered by low-speed centrifugation and washed several times with Tris-NaCl buffer to remove unbound IgG. The gel was then washed with 0.2 M triethanolamine buffer (pH 8.2) and cross-linked with freshly made 20 mM dimethyl pimelimidate in 0.2 M triethanolamine buffer (pH 8.2) and mixed for 3 h at room temperature. The gel was recovered by low-speed centrifugation and treated with aqueous buffered 20 mM ethanolamine in triethanolamine buffer for 5 min to block un-reacted cross-linking agent. The gel was then recovered and washed well with PBS with 0.01% NaN_3 and poured into a 2 mL polystyrene column. Prior to use the column was washed with PBS.

In vitro synthesis of HSA-HHPA conjugates. The HSA-HHPA conjugate was synthesized according to Kristiansson *et al.* (2003). Briefly, HHPA was dissolved in acetonitrile and added to a solution of HSA dissolved in PBS. The molar ratio of added HHPA was 1:10 (HSA:HHPA). The HSA-HHPA solution was incubated at room temperature for 4 h and then transferred to dialysis membrane and dialyzed in PBS with 2 mM EDTA for 5 days.

Evaluation of IAE column. In the evaluation, the IAE column was equilibrated using 2 mL PBS. The sample was applied and the column was then washed with 2 mL PBS. These combined fraction are called the flow-through. The column was eluted with 2 mL 0.1 M glycine buffer (pH 2.0) followed by 2 mL of 10 $\times$ PBS. These combined fractions are called the eluate.

To evaluate the need for a re-circulation of the plasma sample through the column, three replicates of plasma (2 mL)

were spiked with 0.3 nmol HSA–HHPA conjugate and then applied to the IAE column. The samples were drawn through the column by gravity flow and were then re-circulated one, two and three times.

To evaluate the binding capacity of the IAE column, plasma samples (2 mL) were spiked with 0.15, 0.3, 0.6 and 1.2 nmol HSA–HHPA conjugate and then applied to the IAE column. The samples were prepared in duplicate. Each sample was re-circulated three times through the column.

To evaluate the recovery and precision of the IAE column, 11 plasma samples (2 mL) were spiked with 0.3 nmol HSA–HHPA conjugate and then applied to the IAE column. The samples were re-circulated three times through the column.

HHP acid (0.15 nmol) in 2 mL PBS was re-circulated three times on the IAE column to evaluate the affinity of HHP acid on the column.

Application of IAE column. A plasma sample (2 mL) obtained from a worker occupationally exposed to HHPA at an industrial plant was applied to the IAE column. The sample was re-circulated three times through the column. The column was eluted with 2 mL 0.1 M glycine buffer (pH 2.0) followed by 2 mL of 10 × PBS.

Hydrolysis of adducts. Deuterium-labeled HHP acid ($[^2\text{H}_6]$ -HHP acid) was added to the eluates as an internal standard. The HHPA-adducts were hydrolyzed in 0.05 M HCl for 2 h at 100°C and the obtained HHP acid was isolated by solid-phase extraction (SPE) according to a slightly modified procedure (Lindh and Jönsson, 1998) on a C_{18} Bond Elut SPE column (Varian, Harbor City, CA, USA). The column was equilibrated with 0.1 M HCl, and the sample was eluted with 100% methanol and finally evaporated to dryness.

Quantification of HHP acid by LC/MS/MS. The dried samples from the evaluation of the IAE column were dissolved in 100 μL water–methanol (60:40) and transferred to injection vials. HHP acid was then quantified on an LC/MS/MS (API 3000; Applied Biosystems, Foster City, CA, USA) coupled to an HPLC system from Perkin-Elmer (Norwalk, CT, USA) as described by Kristiansson *et al.* (2002). Briefly, samples were analyzed on a C_{18} column (2.1 mm i.d. $\times$ 50 mm, Genesis; Jones Chromatography, Lakewood, CO, USA) in a mobile phase of water and methanol, both containing 0.5% acetic acid. The injection volume was 5 μL , and the flow rate 0.2 mL/min. The mass spectrometric analysis was carried out using selected reaction monitoring in the negative mode and at the transitions m/z 171.0/126.9 (HHP acid) and 177.0/133.0 ($[^2\text{H}_6]$ HHP acid).

Quantification of HHP acid by GC/MS. Plasma from the HHPA exposed worker required lower detection levels of HHP acid than can be achieved by LC/MS/MS, thus this sample was analyzed using GC/MS. The dried eluate was derivatized using PFBB according to Lindh and Jönsson (1997), dissolved in 100 μL of toluene, transferred to injection vials and subsequently analyzed on a VG Trio 1000 MS (Fisons, Manchester, UK) coupled to a Carlo-Erba 8065 GC equipped with an A200S autosampler (Carlo-Erba, Milan, Italy). The instrumental settings of the GC/MS analysis were as described by Lindh and Jönsson (1997).

RESULTS AND DISCUSSION

Evaluation of the IAE column

In previous studies, it has been shown that HSA is the major HHPA-binding protein in plasma (Johannesson *et al.*, 2004). Thus, HSA was chosen as a relevant model carrier protein in the evaluation of the IAE column.

There are several validated quantitative methods described for analysis of protein adducts of HHPA. After an acid hydrolysis of the protein adducts, the corresponding HHP acid is released. The HHP acid can then be analysed using LC/MS/MS or GC/MS. The quantified levels of HHP acid were used as a measure of the HHPA protein adducts and to evaluate the performance of the IAE column. The HSA–HHPA conjugate in this study was synthesized using a molar ratio of 1:10. When 0.30 nmol conjugate was hydrolyzed, 1.13 nmol HHP acid was obtained.

Selection of IgG antibodies

Polyclonal antibodies were raised against HHPA adducts using HHPA coupled to KLH as a carrier protein in rabbits. The rabbits serum was purified on a protein A Sepharose gel, and the resulting IgG pool was further purified using RSA–HHPA conjugates coupled to an NHS–Sepharose column. The anti-HHPA IgG antibodies would be expected to contain a mixture of polyvalent antibodies ranging between low affinity and high affinity toward the antigens (Eisen and Siskind, 1964). The RSA–HHPA conjugate was used to avoid selection of antibodies with affinity towards HSA or other human proteins. To obtain a selection of anti-HHPA IgG antibodies with optimal affinity to the HHPA adducts, the IgG pool were eluted in 12 fractions using glycine buffers at various pH. The obtained fractions were visualized using Coomassie staining on an agarose gel (Fig. 1).

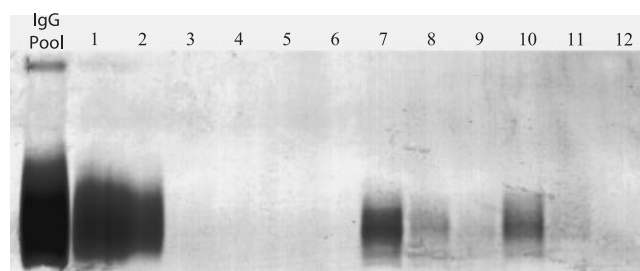


Figure 1. HHPA-specific IgG antibodies eluted from an NHS–Sepharose column coupled with albumin–HHPA conjugate are visualized on an agarose gel. The first lane shows the total IgG pool. Lanes 1–3 show the flow-through and washing of the column. The column was eluted with glycine buffer pH 4.4 (lanes 4–6), pH 3.6 (lanes 7–9) and pH 2.2 (lanes 10–12). The IgG eluates shown in lanes 7–9 were pooled and used for the IAE column.



The antibodies eluted using glycine buffer at pH 3.6 (lanes 7–9, Fig. 1) were selected for the IAE column.

An IAE column was also prepared using the IgG pool eluted at pH 2.2. This column included antibodies with higher affinity directed to the protein adducts. This resulted in an IAE column that retained the HHPA adducts too strongly with lower yields and required harsher conditions for elution (data not shown).

Bonding density

UV absorbance at $\lambda = 280$ nm was measured in the IgG solution before and after coupling to the Protein A Sepharose. The difference was used to calculate the amount of IgGs coupled to the Protein A Sepharose. Approximately 1.5 mg of specific anti-HHPA IgG antibodies were added to 1 mL of the Protein A Sepharose gel. The binding efficiency of IgG to the Protein A Sepharose support was estimated to be between 80 and 90%, yielding a bonding density of 1.1–1.4 mg/mL support.

Effect of re-circulation

Plasma spiked with HSA–HHPA conjugate was applied to the IAE column to evaluate the binding efficiency of the column. Each sample was re-circulated once, twice and three times. The hydrolyzed amount of HHP acid in each eluate was determined using LC/MS/MS. The amount of HHP acid in the eluates increased after each re-circulation, with 56, 59 and 66% of the adducts recovered in each run. Thus, the extent of the binding was improved after each re-circulation. Three re-circulations were used in the further evaluation of the IAE column.

Binding capacity

To determine the binding capacity of the IAE column, plasma spiked with different amounts of HSA–HHPA conjugate was applied to the column. The hydrolyzed amount of HHP acid in each eluate was then determined by LC/MS/MS. The column's capacity to bind the HSA–HHPA conjugate was found to be linear in the range 0.15–1.2 nmol with an equation of the line $y = 2.1x + 0.1$ ($r = 0.99$). In the majority of immuno-affinity columns, non-purified anti-sera or antibodies are used. This could result in reduced bonding density and a less efficient selectivity in the extraction step due to the presence of antibodies having other specificities than the desired (Hennion and Pichon, 2003). In the production of the IAE column described in this paper the anti-serum was not only purified on a Protein A Sepharose support but was also subjected to a second selection step to obtain the HHPA specific IgG antibodies. This would further improve the selective pro-

perties of the IAE column and thus minimize disturbance of co-eluting analytes. It could also increase the binding capacity of the IAE column. Another advantage of using protein A sepharose over other methods using variously activated gels is that protein A Sepharose binds specifically to the Fc region of IgG, thus leaving the antigen binding site maximally exposed (Delaunay *et al.*, 2000).

Recovery and precision

The precision and the recovery of the IAE column was determined by applying 11 plasma samples spiked with HSA–HHPA conjugate to the IAE column. The hydrolyzed amount of HHP acid in each eluate was then determined by LC/MS/MS. The recovery of the HHPA adducts was calculated by comparison with 0.3 nmol hydrolyzed not extracted HHPA–HSA conjugate. The average recovery of HHPA adducts in 11 experiments was 68% with a coefficient of variation (CV) of 7%. The results are shown in Table 1. The binding capacity of the selected IgG pool was considered acceptable and a very good precision were achieved.

The IAE columns affinity toward free HHP acid was also evaluated and it was found that 72% could be found in the flow-through fractions from the column and 24% could be found in the eluate. The data suggests that there is a low affinity for free HHP acid. The HHP acid is not likely to interfere with future analysis of proteins or peptides.

Application of IAE column

Owing to the lower levels of HHPA adducts present in the plasma sample from an exposed worker, the adducts were quantified using GC/MS. Plasma from one worker was analyzed using the method described by Lindh and Jönsson (1997). The plasma contained a total of 16 pmol HHPA adducts. An equal amount plasma was applied to the IAE column and eluted. A total of 13 pmol of HHPA adducts were found in the eluted fraction after adjustment to the recovery of protein on the column. Although there seems to be some loss of the protein adducts as compared with the *in vitro* synthesized HSA–HHPA conjugate, the data suggests that *in vivo* formed protein adducts can also be enriched from a large volume of plasma using the IAE column. In the scope of this study there is no intention to present a quantitative method for adduct determination. In the development of a quantitative method, an internal standard composed of a stable isotope labeled protein conjugate ought to be included.

Durability of the IAE column

The column was regenerated in $10 \times$ PBS to decrease the time the antibodies were exposed to the low pH of

Table 1. Eleven plasma samples (2 mL) spiked with HSA–HHPA conjugate (0.3 nmol) were applied to the IAE column to determine the precision of the column and the recovery. The adducts retrieved in the flow-through and the eluted fraction were hydrolyzed and the HHP acid quantified using LC/MS/MS

HHP acid (nmol)		Recovery of HHP acid (%)	
in eluate	in flow-through + eluate	in eluate	in flow-through + eluate
0.84	1.11	74	98
0.78	1.04	69	92
0.79	1.04	70	92
0.80	1.07	71	94
0.79	1.04	70	92
0.77	1.04	68	92
0.78	1.06	69	94
0.64	0.90	57	80
0.70	1.08	62	95
0.76	1.02	67	90
0.84	1.11	74	98

the glycine buffer. All experiments were performed using the same column. The performance of the column remained unchanged during the course of approximately 100 experiments.

Future use

There are several possible future uses for the IAE column. The mechanistic events behind development of sensitization to HHPA and also other allergic diseases are largely unknown. It has been shown that HSA–HHPA conjugates can induce symptoms in HHPA sensitized workers (Nielsen *et al.*, 1994). It has also been shown that HSA is the major site for *in vivo* formation of hapten-carrier protein adducts in plasma in humans (Johannesson *et al.*, 2001). Also, some modified proteins using proteomics methodology have been identified in nasal lavage from exposed workers (Johannesson *et al.*, 2004). However, only proteins in the high abundance range in plasma or nasal lavage have been identified. To be able to identify modified proteins in the medium or low abundance range that can be mechanistically important, these proteins need to be enriched prior to proteomics identification.

Another use of an IAE column would involve extraction of adducts in plasma followed by enzymatic digest (e.g. trypsin). This would result in adducted peptides that could be detected using LC/MS/MS. This could result in faster and easier methods for adduct quantification. However, to perform absolute quantification of tryptic peptides, synthesized standard peptides and labeled internal standard peptides would be required. It has previously been shown that LC/MS/MS can be used for relative quantification of HHPA adducted HSA tryptic peptides (Kristiansson *et al.*, 2004) in nasal lavage fluids.

CONCLUSIONS

An IAE column was developed for enrichment of low levels of HHPA-adducted protein from large volumes of plasma. Polyclonal antibodies from a rabbit immunized with KLH–HHPA conjugate were purified and selected in two steps to obtain antibodies with adequate affinity. The column was evaluated using plasma spiked with HSA–HHPA conjugate. Protein adducts were extracted from plasma using the column with good precision and recovery.

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