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ISOLATION OF SUBSTANCES FROM URINE BY AFFINITY CHROMATOGRAPHY

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I. INTRODUCTION

Affinity chromatography is a purification technique that relies on the use of specific recognition to affect separation. The method is based on the ability of a ligand (binding protein, enzyme inhibitor, etc.), covalently attached to an insoluble matrix, to specifically bind and separate a desired substance from a mixture. In practice, a substance is usually isolated by passing the mixture through a column containing the immobilized ligand. Ideally, specific interaction with the immobilized agent will prevent or, at least, retard the passage of the desired substance through the column, while non-binding impurities pass through unhindered. The desired substance can then be eluted by any one of a number of procedures that effect dissociation from the immobilized ligand; *e.g.*, a change in column conditions (pH, buffer, ionic strength), or the addition of a soluble compound that competes for binding sites. The technique, either alone or in combination with standard purification procedures, has been used to separate and isolate substances that are otherwise difficult to obtain.

There are a number of excellent books and reviews (*e.g.*, refs. 1 and 2) that deal with the principles, methodology and various applications of affinity chromatography. However, the isolation of urinary substances has not been specifically reviewed. Urine, through its availability and wealth of components, can serve as an excellent source of important and rare physiological substances. Unfortunately, these components are usually present in such low concentrations that they are difficult to isolate by standard methods. On the contrary, the versatility, simplicity and high recoveries possible with affinity chromatography allow large volumes of urine to be

TABLE 1

SUBSTANCES ISOLATED FROM URINE BY AFFINITY CHROMATOGRAPHY

Substance	*Ligand*	*Eluting agent*	*Purification factor*	*Yield (%)*	*Reference*
Enzymes					
Urokinase*	α-Benzylsulfonyl-*p*-aminophenyl-alanine	8% NaCl (pH 7.0)	700	100	3
Urokinase	Lysine	1 *M* NaCl (pH 7.5)	—	100	4–6
Urokinase	D-Hexyl-Nα-(ω-aminocaproyl) homoarginate	0.2 *M* NaCl–6 *M* urea (pH 7.4)	—	80	7
Urokinase	Anti-urokinase	0.2 *M* Na_2CO_3–0.5 *M* NaCl (pH 12)	—	81	8
Kallikrein	Guanidinated inhibitor from bovine organs	0.5 *M* Benzamidine (pH 7.7)	7	~46	9, 10
Kallikrein	Arginine methyl ester	1.0 *M* Arginine (pH 8.1)	87	~19	11
Kallikrein	Aprotinin	1.0 *M* NaCl	328	1.6	12
Pepsinogen I group	Anti-pepsinogen I group	3.0 *M* NaSCN (pH 7.4)	—	—	15
α-Galactosidases	*p*-Aminophenylmelibioside	0.1% Triton X-100 (pH 5.4)	—	—	16
α-Galactosidase	*p*-Aminophenyl-α-D-galacto-pyranoside	250 m*M* NaCl (pH 7.4)	211	4	17
N-Acetyl-β-D-hexosaminidase A	*p*-Aminophenyl-N-acetyl-β-D-thioglucosamine	0.2 *M* Borate (pH 8.0)	4	50	18
N-Acetyl-β-D-hexosaminidase A	Concanavalin A	0.5 *M* L-Methyl glucoside (pH 7.0)	1000	13	19
Arylsulfatase A	Psychosine sulfate	0.1% Triton X-100 (pH 5.7)	175	13	20, 21
Arysulfatase A	Sulfopropyl radical	50 m*M* NaCl (pH 5.0)	3500	7	22
α-L-Iduronidase	Heparin or dermatan sulfate	NaCl gradient (pH 6.0)	—	—	24

Proteins					
Transferrin	Anti-transferrin	1.0 *M* Glycine·HCl (pH 2.8)	–	–	26, 27
Hemagglutinating antibodies	O-Antigen	Physiological saline	16	–	28
Retinol-binding protein	Prealbumin	Distilled, deionized water (pH 8)	–	31	29
Hormones					
Human chorionic gonadotrophin	Concanavalin A	0.2 *M* Methyl α-D-glycopyranoside (pH 7.4)	–	–	30
Insulin, proinsulin	Anti-insulin	1.0 *M* Acetic acid–0.3% BSA	–	100	31
Colony-stimulating factor	Concanavalin A	α-Methyl-D-mannopyranoside or α-methyl-D-glucopyranoside gradients	6–9	50	33
Epidermal growth factor	Anti-mouse epidermal growth factor	0.55 *M* Formic acid	–	–	34
Miscellaneous ingredients					
Basement membrane antigens	Anti-human glomerular basement membrane	0.1 *M* Glycine·HCl (pH 2.8)	–	–	35
Trypsin-inhibitor	Trypsin	0.02 *M* HCl, 0.3 *M* NaCl, 0.01 *M* $CaCl_2$	–	85	37
D-Lysergic acid diethylamide	Anti-lysergide	0.01 *N* HCl	–	70–80	38
Ethynyl steroids	Silver-sulfoethyl cellulose	Saturated NaCl–methanol	–	~100	39
Spermine and spermidine	Copper-loaded resin	5.9 *M* Ammonia	–	80	40

* Although the data refer to enzyme isolated from a tissue culture medium, the authors indicate that the procedure is applicable to urine.

conveniently processed, thus permitting isolation of these substances in practical amounts.

Here, we will review the uses of affinity chromatography for the purification and characterization of various biological substances from urine. (A summary is provided in Table 1.) Moreover, we will briefly discuss some analytical applications of affinity supports for the detection and quantification of various materials in urine. Finally, we hope to draw attention to the versatility and usefulness of affinity isolation procedures and to the use of urine as a source of fine reagents. We have also included some separations that involve other mechanisms, such as ligand exchange, when these gave specific isolation of important constituents of urine.

II. PREPARATIVE APPLICATIONS

(A) Enzymes

Urokinase is a protease that has therapeutic value as a thrombolytic agent. It is present in such small quantities in human urine that isolation of significant amounts by classical procedures is impractical. The enzyme, however, has been purified from tissue culture media[3] or urine[3–6] by affinity chromatography using the ligands α-benzylsulfonyl-*p*-aminophenylalanine (BAPA) or lysine covalently linked to a matrix such as Sepharose. Albumin contamination was removed by a separate affinity procedure before the mixture containing urokinase was loaded onto the BAPA–Sepharose column. The enzyme was eluted with 8% NaCl in phosphate buffer in 100% yield with a purification factor of approximately 700. Urokinase has also been obtained from urine in 100% yield using a lysine–Sepharose conjugate and 1 *M* NaCl as the eluting agent. The enzyme has been isolated (80% yield) using D-hexyl-Nα-(ω-aminocaproyl) homoarginate as the binding ligand and 0.2 *M* NaCl–6 *M* urea as the eluting agent[7], and also from an immunosorbent column after desorption with 0.2 *M* Na_2CO_3 containing 0.5 *M* NaCl (81% yield)[8].

Kallikreins are endogenous proteases that rapidly and specifically liberate kinins from precursor kininogens in plasma. These enzymes are found in urine and have been isolated by affinity chromatography. Guanidinated inhibitors from bovine organs have been coupled to CM-cellulose via the azide and used to isolate kallikreins from porcine urine[9,10]. The kallikrein–inhibitor complexes were dissociated with 0.5 *M* benzamidine to give a 46% yield of enzyme purified approximately 7-fold. Rat urinary kallikrein was isolated by a procedure which included an affinity chromatography step[11]. An L-arginine methyl ester–Sepharose conjugate specifically bound the protease which was subsequently eluted with 1.0 *M* arginine. The enzyme, obtained in 19% yield, underwent an 87-fold purification. The purity of the enzyme was established by polyacrylamide electrophoresis and electrofocusing, and its molecular weight was determined by gel filtration. The enzyme was further characterized through a study of its activity with natural and synthetic substrates and inhibitors. Rat urinary kallikrein was also isolated with a purification factor of 328 in 1.6% yield using a procedure consisting of four steps: DE-32 chromatography, affinity chromatography on a Bio-Gel aprotinin column, and gel filtration over Sephadex G-100, coarse and superfine[12]. The physical and biological activities of this enzyme preparation were investigated and reported.

Human pepsinogen I group materials were isolated from human urine[13,14];

e.g. through a combination of ion-exchange chromatography, and two immuno-adsorption steps[15]. Fractions obtained from the ion-exchange step were applied to an anti-pepsinogen I group–Sepharose column and desorption was carried out with 3.0 *M* NaSCN. These fractions were then applied to an antihuman serum column to remove trace contaminants. The biochemical and immunochemical properties of the pepsinogen I group material isolated from urine were compared with those of the pepsinogen I group from gastric mucosal extracts and found to be identical. Apparently, the individual proteases of the group I material possess the same antigenic determinants.

An approach to the control of enzyme deficiency diseases that has aroused considerable attention has been the use of enzymes in replacement therapy. Hopefully, the administration of the deficient enzyme will be of therapeutic value. For this reason, the isolation, purification and characterization of human glycosphingolipid hydrolases has become an area of keen interest. Fabry's disease is caused by a deficiency of ceramide trihexosidase, an acid α-galactosidase. Plasma and urine have served as sources for α-galactosidases isolated through affinity procedures[16]. The ligand, *p*-aminophenylmelibioside, linked via a bridging group to agarose was used to isolate multiple forms of ceramide trihexosidase activity from human urine. The enzymes desorbed from the affinity column with 0.1% Triton X-100 showed elution profiles very similar to those obtained from plasma. The occurrence and properties of these enzymes in normals and patients with Fabry's disease were studied. A subsequent publication, described the isolation of α-galactosidase from normal human urine by a procedure that involved ammonium sulfate precipitation, treatment with cetylpyridinium chloride, precipitation by ethanol, chromatography on CM-cellulose, affinity chromatography on a *p*-aminophenyl-α-D-galactopyranoside–Sepharose column, and finally chromatography on Sephadex G-200 (ref. 17). The purified enzyme was used to raise antisera in rabbits. The antiserum, in turn, was used to demonstrate the absence of a specific α-galactosidase (the immunogen) in urine and kidney preparations from a Fabry patient. These data suggest that the disease is due to total absence of the protein and not to an enzymatically inactive protein. They also suggest that immunological complications may arise in the course of enzyme replacement therapy, since the immune mechanism may respond to the exogeneously provided enzyme.

Tay-Sachs' and Sandhoff's diseases are caused by deficiences of the A and the A and B isoenzymes of N-acetyl-β-D-hexosaminidase, respectively. In combination with other procedures, an affinity chromatography step, using a conjugate of succinylated diaminodipropylaminoagarose and *p*-aminophenyl-N-acetyl-β-D-thioglucosamine, was used to partially purify the A isoenzyme from urine[18]. The enzyme was eluted with 0.2 *M* borate buffer but was contaminated with three to six other proteins, leading the authors to suggest that the affinity step be combined with still other procedures if a homogeneous product is desired. Interestingly, the B isoenzyme was only slightly retarded on the affinity support even though both isoenzymes have similar activities toward a substrate resembling the bound ligand. The A isoenzyme has recently been purified from human urine with a 1000-fold increase in specific activity by a combination of ion-exchange and affinity chromatography[19]. A column of concanavalin A–Sepharose was used in the latter procedure.

A deficiency of the enzyme arylsulfatase A is diagnostic for the lipid storage

disease, metachromatic leukodystrophy. This enzyme was purified from human urine to homogeneity as judged by electrophoresis at pH 8.9, as well as electrophoresis in the presence of 8.0 *M* urea at pH 2.9. The procedure used an affinity chromatography step on a Sepharose–psychosine sulfate conjugate combined with such standard techniques as precipitation, acetone fractionation, Sephadex chromatography and preparative gel electrophoresis[20,21]. Enzyme activity was eluted from the affinity support with 0.1% Triton X-100. The same enzyme was later purified 3500 times in a 7% yield from human urine[22]. This preparation was likewise judged to be essentially homogeneous by a number of criteria, including electrophoresis at two pH values, formation of a single band upon electrophoresis in a sodium dodecyl sulfate gel, and formation of a single precipitin line in Ouchterlony double diffusion studies. In addition to standard protein purification procedures, two affinity steps were included in the isolation protocol. The first of these involved chromatography on sulfopropyl–Sephadex to extract the enzyme, while the second affinity chromatography step used an antialbumin–Sepharose conjugate to remove carryover contamination. The physical and immunochemical properties and the catalytic characteristics of this enzyme with natural and synthetic substrates were examined. Sphingomyelinase was also isolated from urine with affinity columns containing arylphosphorylcholine[21] or sphingosinephosphorylcholine[23].

Hurler's syndrome, a genetic disorder of mucopolysaccharide metabolism, results from a marked deficiency of α-L-iduronidase, which causes an excessive lysosomal accumulation of mucopolysaccharides. Excessive amounts of mucopolysaccharides have been reduced to normal levels in tissue culture through the addition of Hurler-corrective factor (α-L-iduronidase). Thus, the enzyme is of practical importance as a candidate for replacement therapy. It is present in human urine and has been isolated by a protocol that includes an affinity chromatography step[24]. A heparin– or dermatan sulfate–Sepharose conjugate used as the affinity support not only retained α-L-iduronidase, but also separated α-L-iduronidase with Hurler-corrective ability from an α-L-iduronidase devoid of this capability. The corrective and non-corrective forms of the enzyme had similar kinetic characteristics, but differed somewhat in their ability to bind to various lectins and had substantially different molecular weights. The authors speculated that the non-corrective form of the enzyme may be either a precursor or degradation product of the corrective form. A similar corrective factor for Hunter's syndrome was purified from human urine and characterized[25]. Chromatography on an antialbumin–Sepharose column was used during the isolation procedure to remove residual albumin contamination that could not be eliminated by conventional techniques.

(B) Proteins

An immunosorbent prepared by polymerizing antisera to transferrin with glutaraldehyde has been used in conjunction with standard procedures to isolate transferrin from normal human urine[26,27]. Urinary transferrin, eluted from the immunosorbent with 1.0 *M* glycine·HCl, was homogeneous by electrophoresis and immunoelectrophoresis and was similar in physical and immunochemical properties to serum transferrin. Likewise, an immunosorbent prepared from sheep erythrocytes and O antigen has been used for a 16-fold concentration of hemagglutinating antibodies from urine[28]. The antibodies were eluted in a small amount of physiological saline.

Urine from patients with tubular proteinuria is a good source of low-molecular-weight plasma proteins. For example, human retinol-binding protein (vitamin A transport protein) was highly purified from urine by a procedure that included chromatography on a prealbumin–Sepharose column[29]. (In plasma, retinol-binding protein circulates firmly bound to thyroxine-binding prealbumin, but can be dissociated at low ionic strength.) Two forms of the protein, differing in vitamin A content and ability to bind to prealbumin, were isolated by this approach. The purer fraction, which bound tightly to the affinity support, was immunochemically and physically characterized and found to be very similar to retinol-binding protein isolated from serum.

(C) Hormones

A column containing a concanavalin A–Sepharose conjugate was used to isolate and obtain structural information on human urinary chorionic gonadotrophin[30]. The glycoprotein bound through its carbohydrate portion and could be eluted from the support with 0.2 *M* methyl α-D-glucopyranoside.

An immunosorbent prepared from guinea pig antisera to insulin and Sepharose was used to extract quantitatively both insulin and proinsulin from urine[31]. The hormones were subsequently eluted from the affinity support with a 1.0 *M* acetic acid solution containing 0.3% bovine serum albumin. The isolation procedure was convenient and, in many respects, superior to conventional methods for isolating insulin from biofluids.

Colony-stimulating factor (CSF) is a term applied to material that promotes the formation of granulocyte and/or macrophage colonies in culture. Since this material was suggested to be glycoprotein in nature, a concanavalin A–Sepharose conjugate was used to isolate and partially purify the substance[32,33]. The affinity matrix was eluted with a linear gradient of either α-methyl-D-mannopyranoside or α-methyl-D-glucopyranoside to give CSF in 50% yield with a six- to nine-fold increase in specific biological activity[33]. A study of the physical properties of the partially purified preparation suggested the activity was associated with a heterogeneous population of molecules.

Epidermal growth factor is a polypeptide hormone that causes stimulation of epidermal growth and keratinization. An immunosorbent column prepared from Sepharose and rabbit antiserum to mouse epidermal growth factor was used to isolate and partially purify a substance from human urine that is biologically and immunologically similar to the mouse hormone[34].

(D) Other urine ingredients

Rabbit antisera to human glomerular basement membrane have been coupled to Sepharose and used to isolate basement membrane antigens from normal human urine in a single-step procedure[35]. The immunosorbent, eluted with 0.1 *M* glycine·HCl buffer, provided material for characterization studies.

Affinity chromatography has also been used to isolate trypsin inhibitors from urine[36,37] using a trypsin–Sepharose conjugate in a batch procedure followed by elution with 0.02 *M* HCl containing 0.3 *M* NaCl and 0.01 *M* $CaCl_2$. Material isolated in this manner gave two active fractions upon subsequent chromatography on DEAE-cellulose. This finding led the authors to speculate that the heterogeneity of the

inhibitors is not caused by the extraction process or degradation of a single inhibitor molecule by a urinary enzyme, but results because several trypsin inhibitors are actually present in urine.

Affinity supports have been used to isolate drugs and drug metabolites from urine. For example, D-lysergic acid diethylamide added to normal human urine has been recovered in a 70–80% yield through use of an immunosorbent column and elution with 0.01 *N* hydrochloric acid[38]. Columns of silver–sulfoethyl cellulose bound an entire class of compounds, the ethynyl steroids[39]. The columns were used to advantage in the purification of norethynodrel metabolites from the urine of a beagle.

Metal-loaded resins were used to separate and concentrate aliphatic diamines and polyamines from urine[40]. Ten-milliliter urine samples containing 1.0 mg each of added spermine and spermidine hydrochlorides were passed through a copper-containing column and eluted with ammonia, giving well-separated, well-defined peaks for spermine and spermidine in about 80% yield.

III. ANALYTICAL APPLICATIONS

Affinity supports have also found considerable application in the detection and quantification of materials in urine in addition to their use in preparative schemes. A brief review of some of the analytical applications will be given to indicate the variety of assays that have been developed. A more comprehensive review can be found elsewhere[41].

Urinary gonadotrophins have been assayed by a number of radioassay techniques that employ immunosorbents to separate bound and free fractions. Follicle-stimulating hormone (FSH) assays were conducted on 24-h urine specimens using both a radioimmunoassay, in which antibody to pituitary FSH was adsorbed to bentonite particles, and a bioassay procedure[42]. There was a significant correlation between the values obtained from both assays. Assays for luteinizing hormone and FSH in urine specimens were developed using a solid-phase double antibody system[43] and by procedures relying on polymerized first antibody[44]. Nylon microtiter plates have served as a solid-phase support for adsorbed antibody and have been used to assay epidermal growth factor in urine[45]. Other proteins such as α-N-acetylglucosaminidase mutants[46], and immunoglobulin E [47,48] have been measured in urine with radioimmunoassay procedures based on immunosorbents in which the antibody was covalently linked to the solid phase.

In addition to proteins, low-molecular-weight substances in urine have been assayed using affinity supports. An antiserum raised against estriol-16-glucoronide was incorporated in a polyacrylamide gel and used to assay the estriol derivative directly in maternal urine, *i.e.*, without prior hydrolysis or purification[49]. Hemagglutination-inhibition assays have been developed for various drugs in urine[50].

IV. SUMMARY

The applications of affinity chromatography for the isolation and purification of physiological substances from urine have been reviewed. The use of affinity supports for the detection and measurement of various urinary components has also

been briefly examined. These examples illustrate the usefulness of urine as a source of fine reagents and the versatility, simplicity and practicality of affinity isolation procedures.

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