The separation of peptides from amino acids in urine by ligand exchange chromatography*

Normal human urine contains more than 1 g of bound amino acids in 24 h. Little is known about the origin or significance of these peptides although pathological peptiduria has been demonstrated in certain diseases. This report describes a technique, utilizing ligand exchange chromatography[1], for separating the peptides of urine from the complex mixture of amino acids. The principle on which this is based is that a transition metal is complexed with a cation exchange resin and the metallic ions can then shed their solvation shells and will form stable complexes with amino acids. The metal–resin complex will continue to extract amines from very dilute solutions until the coordinative valences of the metal ions are saturated[2]. SIEGEL AND DEGENS[1] employed this principle to concentrate the free and bound amino acids in sea water and obtained amino acid recovery rates of 100 %. The procedure described here is based on that of FAZAKERLEY AND BEST[3] who employed column chromatography with Sephadex bonded with copper, under alkaline conditions. They reported the separation of amino acids from peptides with recovery rates approaching 100 % for the amino acids. Peptide recovery rates, however, were not reported. They suggested that the use of a resin with a stronger affinity for copper than Sephadex would be more suitable for the study of peptide recoveries because of a greater retentive capacity for the metal ions.

The resin employed in this study was Chelex 100 (Biorad, Calif.) sodium form, mesh size 50–100. The active sites of this resin are iminodiacetic groups which have a strong affinity for the transition metals. The copper bonding is so strong that the complexed resin can be used in the presence of strong ionic solutions without causing the displacement of copper from the resin by other ions.

Methods

The resin was washed with ten bed volumes of deionized water, added to a saturated solution of copper sulfate and stirred at 4° for 24 h. Subsequently the supernatant copper solution was decanted and the resin washed with deionized water until the washings showed no free copper on the addition of sodium diethyldithiocarbamate. The resin was then suspended in 0.01 *M* sodium tetraborate buffer at pH 11 and 1 *N* sodium hydroxide was added to bring the suspension finally to pH 11. The prepared copper–Chelex resin was packed into a column (1.3 × 12 cm) and washed with a further 25 ml of 0.01 *M* borate buffer at pH 11 at an elution rate of 0.5 ml per min.

Samples containing 0.03 to 0.07 mmole (5–10 mg) of amino acids and a similar quantity of peptides were taken to pH 11 with 0.1 *N* sodium hydroxide and then directly applied to the top of the resin. Elution was continued by gravity at 0.5 ml per min using 0.01 *M* borate buffer at pH 11 until 50 ml of eluate had been obtained. The column eluate was maintained at 0° and collected in a container with sufficient 1 *N* hydrochloric acid at 0° to neutralize the buffer. When peptides were present in the sample, copper appeared in the eluate and removal of this could be achieved by the method recommended by FAZAKERLEY AND BEST[3]. The method requires that copper

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be removed by a methanolic solution of sodium diethyldithiocarbamate (3.5 g in 10 ml); the mixture is shaken and then allowed to stand for some minutes and the brown derivatives and excess reagent are then removed by extraction with 500 ml of chloroform followed by 2 × 200 ml portions. The final solution is taken to dryness *in vacuo* at —60°. Finally, the dried material is redissolved in deionized water to reconstitute the original sample volume.

Amino acid analyses

All amino acid analyses were performed on a Beckman Spinco 120B Amino Acid Analyzer employing physiological resins according to the method of BENSON AND PATTERSON[4]. The use of high-sensitivity cuvettes permitted the analysis of as little as 0.01 micromoles of amino acid. Analyses were performed upon aliquots of the original sample and of the column eluant before and after hydrolysis. Hydrolysis was carried out in sealed glass containers with 6*N* hydrochloric acid at 100° for 22 h. The containers were filled with argon and subsequently evacuated before sealing. Following hydrolysis the sample was filtered to remove humin and the samples then frozen and taken to dryness *in vacuo* at —60°. The sample was then reconstituted to its original volume with 0.2 *N* pH 2.2 citrate buffer.

Results

The method was first assessed using pure mixtures of amino acids or peptides. No α-amino acids appeared in the column eluate, while dipeptides and polypeptides were not retained by the resin and were recoverable from the eluate along with large quantities of copper. Recovery rates were greater than 90 % for diglycine, triglycine, tetraglycine, glycylproline, prolylhydroxyproline and leucylglycylphenylalanine, whether these peptides were present in pure solution or admixed with solutions containing amino acids. Similar results were found for larger physiological polypeptides such as vasopressin, angiotensin II, glutathione and glucagon and also for albumin which had been predialyzed for three days. Uric acid, urea, sugars and creatinine also passed through the column under the conditions stated.

Fig. 1 shows a representative set of long column chromatograms of the acidic and neutral amino acids from an untreated normal urine (No. 1) and after passage through Chelex (No. 2), also the hydrolyzed control urine (No. 3) and the hydrolyzed column eluate (No. 4). From the diagram it willl be seen that the resin removed almost 100 % of the normal α-amino acids found in urine but did not retain many of the substances, mostly of unknown composition, which run before 4-hydroxyproline in this system. In this sample very few, if any, ninhydrin positive peptides were eluted from the column after 4-hydroxyproline or if there were any present they were not present in sufficient quantity to be detected by the system employed. By contrast, the chromatogram of the column eluant following hydrolysis, shown in Fig. 1 No. 4, shows all the physiological amino acids found in normal protein hydrolysates and the chromatogram is very similar to that of the hydrolyzed control urine (Fig. 1 No. 3).

The amino acid contents of the samples shown in Fig. 1 are presented in Table I. The poor recovery rates shown for the basic amino acids may be explained by the fact that peptides containing basic amino acids have additional free amino groups which will have a strong affinity for the copper on the resin and these may be retained by the resin. Poor recovery rates for those amino acids present in low concentrations are due

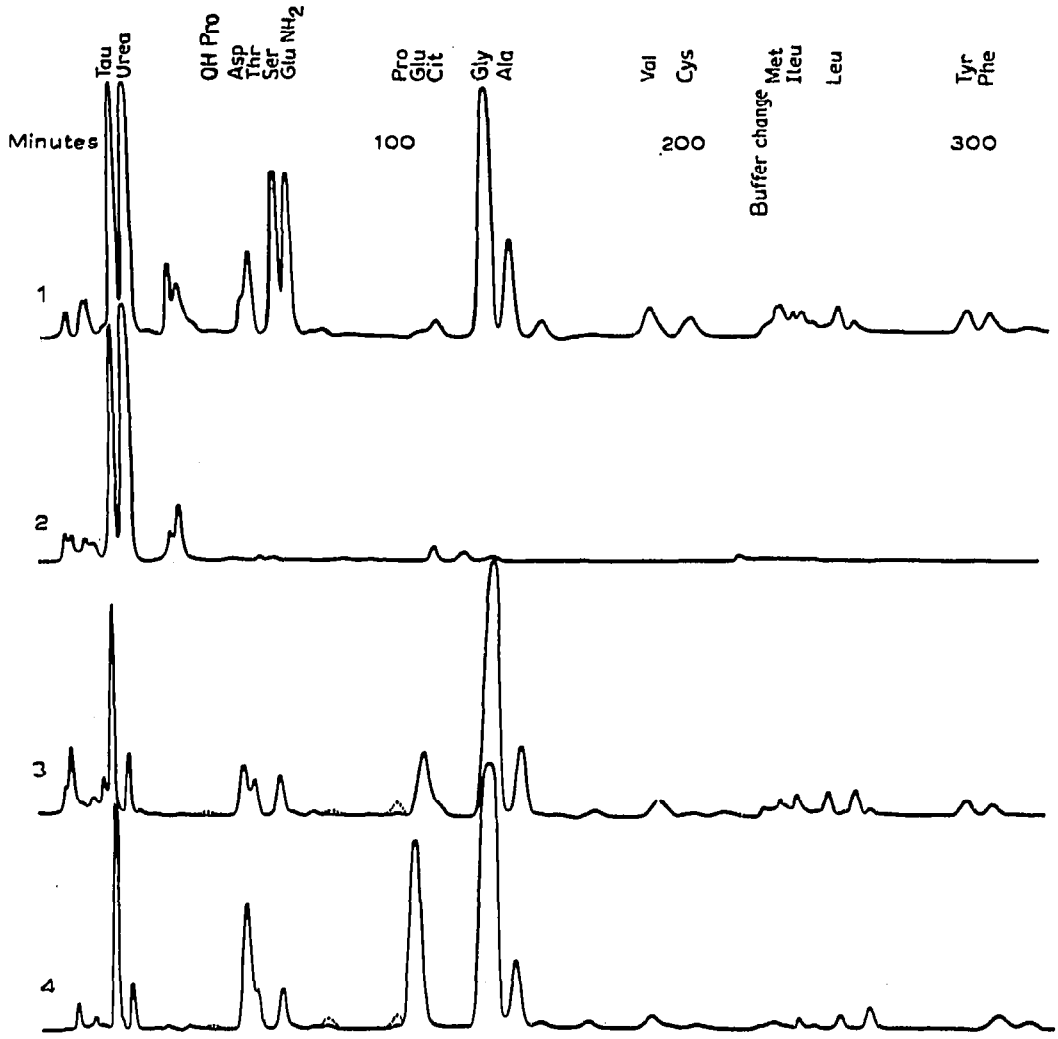

Fig. 1. The acidic and neutral amino acids of urine processed by ligand exchange chromatography before and after hydrolysis. (1) 0.1 ml control urine, unhydrolysed; (2) 0.1 ml urine after passage through resin, unhydrolysed; (3) 0.1 ml control urine, hydrolysed; (4) 0.1 ml urine after passage through resin, hydrolysed. Solid line represents reading at 570 Å. Dotted line represents reading at 440 Å.

to the inherent limits of sensitivity imposed by the analytical system employed. The apparent high recoveries of threonine are due to the progressive hydrolytic destruction of the free amino acid in the control urine[5]. An alternative explanation may be the presence of a substance in the control urine which runs in the same position as the pure amino acid during column chromatography and which is subsequently destroyed by acid hydrolysis: alteration of the conditions of hydrolysis did not materially alter the results.

The reason why amino acids are retained by the column and peptides are not is unclear, since both amino acids and peptides are possessed of free amino groups. The fact that peptides will strip copper from the resin seems to indicate that peptide bonds have an even stronger affinity for the metal than do the iminodiacetic groups and, further, that the peptide–copper complex is then configurationally unable to form a further ligand bond between its free amino groups and the copper on the resin. It will be seen in Fig. 1. that the β-amino acid taurine is not retained by the column.

TABLE I

FREE AND BOUND AMINO ACIDS (μmol/ml) IN NORMAL HUMAN URINE FOLLOWING LIGAND EXCHANGE CHROMATOGRAPHY

The figures shown in Columns 1 through 4 represent the amino acid content of the samples with the same number as shown in Fig. 1.

Amino acid or compound	*Control urine, free amino acids No. 1*	*Urine, following Chelex 100 No. 2*	*Hydrolyzed urine, total amino acid No. 3*	*Urine following Chelex, hydrolyzed peptide amino acid contents No. 4*	*Control urine, peptide amino acid content* No. 5*	*Percentage of recovery (%) No. 6*
Taurine	2.060	2.107	2.506	2.032	0.446	
Phenylacetyl-glutamine	0.204	0.170	0.279	0.019	0.075	
Unknown	0.304	0.290				
Hydroxyproline	—	—	0.230	0.080	0.230	34
Aspartic acid	+	—	1.157	0.830	0.920	90
Threonine	0.202	—	0.357	0.227	0.155	146
Serine	0.390	—	0.699	0.184	0.309	60
Glutamine	0.688	—	—			
Proline	—	—	0.442	0.345	0.442	78
Glutamic acid	0.018	—	2.925	2.074	2.219	93
Citrulline	0.050	Trace	—	—	—	
Glycine	1.664	Trace	11.115	8.897	9.451	94
Alanine	0.286	—	0.744	0.438	0.458	96
α-Aminoadipic acid	0.066	—	0.102	0.026	0.036	72
α-Amino-*n*-butyric acid	0.015	—	0.055	0.050	0.040	125
Valine	0.106	—	0.231	0.124	0.125	100
Half cystine	0.150	—	0.100	0.092	+	?
Methionine	0.052	—	0.027	0.021	—	?
Isoleucine	0.046	—	0.072	0.053	0.026	204
Leucine	0.048	—	0.167	0.103	0.119	87
Tyrosine	0.062	—	0.188	0.130	0.126	103
Phenylalanine	0.062	—	0.104	0.044	0.042	105
allo-Hydroxylysine	0	—	0.086	0.057	0.086	66
Hydroxylysine	0.092	—	0.091	0.046	—	?
γ-Aminobutyric acid	0.026	—	0.179	0.065	0.153	58
Ornithine	0.058	—	0.093	0.041	0.035	
Lysine	0.158	—	0.646	0.261	0.488	53
1-Methylhistidine	0.700	—	0.738	—	0.038	0
Histidine	1.241	—	1.301	0.024	0.060	40
3-Methylhistidine	0.492	—	0.478	0.049	—	?
Arginine	0.008	—	0.081	0.033	0.073	45
TOTALS				14.294	15.631	91

* Column 5 is derived by subtracting free from total amino acids (column 3 minus column 1).

Discussion

Techniques for the separation and identification of urinary amino acids and peptides have developed rapidly in the last two decades. A review of the subject was published in 1962 by SKARZYNSKI[6]. STEIN[7] has reported on the quantities of the bound amino acids in normal urine as released by acid hydrolysis. The figures shown in Table I are in close agreement with his results.

KING[8] has collected column eluate from a standard 22 h chromatogram of normal urine in two hourly fractions. Hydrolysis of these fractions and subsequent amino acid analysis then demonstrated the presence of bound amino acids in all portions of the chromatogram, even when it was thought that all of the ninhydrin positive peaks were definitively identified. In other words, peptides are eluted during the whole period of the standard physiological amino acid chromatogram of normal urine and they are mostly present either in quantities insufficient to be detected by the system or are not primarily ninhydrin positive. The results reported here confirm this. In 1955, WESTALL[9], starting with urine volumes of 100 l, demonstrated the presence of 22 ninhydrin positive peptides in normal urines. In this study we have analyzed only those peptides detectable in 0.25 ml of urine. It is likely that a more concentrated preparation of urine will reveal many more ninydrin positive peaks on a normal urinary amino acid chromatogram. Study of this is under progress.

The technique presented in this paper is based on the principle of ligand exchange chromatography[10]. The method can be utilized as a preparatory step in the isolation and identification of urinary peptides.

Department of Pediatric Microchemistry,
University of Colorado Medical Center,
Denver, Colo. (U.S.A.)

NEIL R. M. BUIST*
DONOUGH O'BRIEN

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* Present address: Assistant Professor of Pediatrics, University of Oregon Medical School, 3181 S.W. Sam Jackson Park Road, Portland, Oreg. 97201, U.S.A.