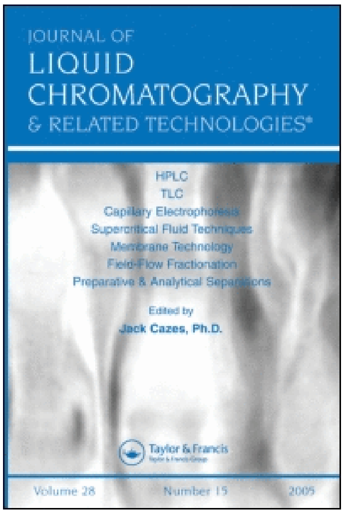

Journal of Liquid Chromatography & Related Technologies
Publication details, including instructions for authors and subscription information:
http://www.informaworld.com/smpp/title~content=t713597273

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To cite this Article Dutrieu, J. and Delmotte, Y. A.(1987) 'A Rapid Preparative Procedure for the Treatment of Free Urinary Catecholamines', Journal of Liquid Chromatography & Related Technologies, 10: 4, 715 — 729
To link to this Article: DOI: 10.1080/01483918708069021
URL: http://dx.doi.org/10.1080/01483918708069021

JOURNAL OF LIQUID CHROMATOGRAPHY, 10(4), 715-729 (1987)

A RAPID PREPARATIVE PROCEDURE FOR THE TREATMENT OF FREE URINARY CATECHOLAMINES

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ABSTRACT

We report a preparative and liquid chromatographic method for the determination of free urinary catecholamines in urine. An extraction column device (1) was used to isolate norepinephrine, epinephrine on a weak cation exchange resin (Bio-Rex 70) using a table centrifuge forcing the sample through the resin material.
The preparation time for a 8 sample size was 50 minutes. The extraction recovery was 80.33 % and 80.89 % for norepinephrine (NE) and epinephrine (E). The curve was linear up to 400 ug/24 H.
The within-run CV was 3.84 % and 4.22 % for NE and E; the between-run CV was 5.58 % and 6.27 % respectively for NE and E. The mobile phase consisted of a 90 % phosphate buffer (0.05M $NA2HPO4$) and 10 % methanol adjusted to pH.3.8.
Chromatographically well separated on a C-8 reverse phase column, the eluent was monitored with a coulometric electrochemical detector set up at 0.40 V.

INTRODUCTION

The analysis of specific compounds in complex media such as body fluids, extract from biological tissues, foodstuffs, water implies their removal from the system, their purification and their concentration. The extraction of the compounds of interest is commonly performed with the traditional pre-filled extraction columns introduced by BIO-RAD Laboratories(2). The resin materials are now highly purified, narrowly sized and loose, and are available in a very wide variety of types dedicated for sensitive laboratory applications. We dedicated for the purification and the extraction of free urinary catecholamines, a special column device filled up with weakly acidic ion exchange resin(BIO-REX 70),in combination with a table centrifuge electronically modified to insure a stable preset rotation speed.

The need for rapid analysis of catecholamines and their derivatives arises from their biological importance (3,4). The clinical significance of these substances is well recognized. A suitable preparative and chromatographic technique (5,6) as well as a specific detection (7,8,9,10,11,12) are powerful tools for their clinical analysis in view of the diagnostic purpose.

The column used as preparative tool (1) was worked in a polycarbonate bar (Figure 1). It is constitued of two parts : the upper part - a 3.5 mL reservoir situated above a 2 mL resin funnel filled up with a selected chromatographic media (BIO-REX 70), and limited to its base by a pourous polyethylene support disc, and the lower part - the collection reservoir of the different elution solvents. The two parts fit together to form a cylinder tube, comparable in size with the red top blood tybe from BECTON-DICKINSON and

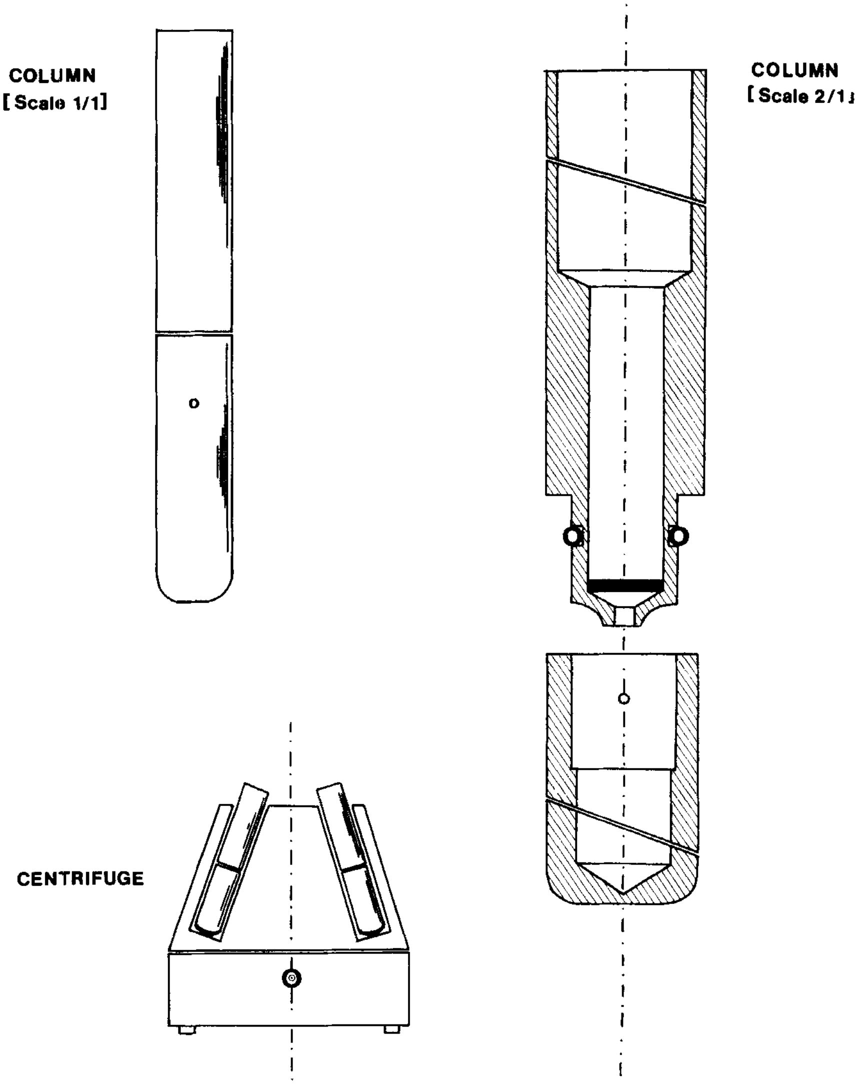

FIGURE 1.

Extraction column device dedicated for buffered sorbent as Bio-Rex 70.

TERUMO companies and compatible with all the existing type of bench centrifuges. We work with a picolo centrifuge model which has been electronically stabilized (1) by integration of the tension between two magnetic captors fixed on the rotor and the stator, in comparison with a settled reference tension.
The centrifuge motor was transformed into a servomotor by mounting a triac which is commanded by the error signal. The reference tension was stated in order to obtain a wide speed range comprised between 200 and 1000 RPMS.

MATERIALS

We used a 850 Du Pont Liquid Chromatograph equipped with a 6 port injection valve Model 7125 (Rheodyne, U.S.A.) placed in a temperature controlled column compartment. A reverse phase column was used, packed with Zorbax C-8 Golden serie 7 mm i.d and 100 mm length. The electrochemical detector used, was constitued of a low flow-through cell containing the reference electrode (Pd), a counter and two porous graphite electrodes as working electrodes : the first one was the "dummy" and the second set up at 0.40 V.
The total cell volume was less than 5 uL. The guard cell consisted of a porous graphite electrode, fixed at 0.70 V which was connected between the pump and the injection valve.
The mobile phase used for the separation was 10 % methanol and 90 % phosphate buffer (0.05M NA2HPO4 and 2.4 10-3M sodium octylsulfonate). The pH was adjusted to 3.8 with diluted phosphoric acid. The solution was filtered through a 0.22 uM membrane filter (Millipore, U.S.A.). Epinephrine (E), Norepinephrine (NE), 3,4-dihydroxybenzylamine hydrobromide(DHBA) were obtained from JANSSENS CHIMICA, Beerse, BELGIUM.

The stock catecholamines standard solutions (10 mg/L) were prepared in 0.05M HCL and stored at 4°C. Working solutions (1 ug/mL) were prepared just before use by diluting 10 mL of the stock standard solution to 100 mL with water.

The cation exchange resin, BIO-REX 70 50-100 mesh was provided from BIO-RAD Laboratories (Richmond, CA).

The lyophilized urine control used (Level II : Lot No 11200), was from E.C.S. (Anahein, CA).

The octane sulfonic acid (sodium form) was obtained from Eastman Kodak (Rochester, N.Y.).

METHOD

To 1 mL of acidified urine, we added 250 uL of the internal standard solution (1 ug/mL DHBA), 4 mL of 0.05M phosphate buffer pH 6.5. The pH was adjusted to 6.5 +/- 0.05 by addition of diluted NAOH.

The columns were arranged on a rack and packed with the analytical grade resin prepared in the phosphate buffer on which the sample will be applied. The volume of the slurry should be approximately twice of third the final volume of the packed resin bed, in order to remove any trapped air bubble during the column packing. The columns were centrifuged at 200 rpms for 30 to 50 s. Resin was added until the bed volume was about 2 mL. The columns packed in the same conditions were ready to be used.

The columns were rinsed with 3.5 mL of 0.05M phosphate buffer to insure complete re-equilibration of the resin. Samples were poured onto the columns and centrifuged for about 60 s at 200 rpms/min. Rinse the columns with 2 mL water. Wastes were removed (They could be collected if the metabolites analysis are requested). The reservoir was filled up with 2 X 2.5 mL Boric acid 4 % and centrifuged at 200 rpms for 45 s. The eluents were collected and mixed in 10 mL glass tubes. We added to the eluates 50 mg of alumina and adjusted the pH to 8.5 (13) with 3M Tris buffer solution. The tubes were mixed and placed on a mechanical shaker for 10 minutes. The supernatant was removed by vacuum aspiration. The alumina was washed with 3 mL water and allowed to sediment. The upper phase was aspirated to dryness. The catecholamines were eluted from the alumina with 300 uL of 1M acetic acid and mixed again for 10 minutes to insure the complete elution of the catechols. The supernatant was filtered through disposable Acrofilter LC 13 (0.45 um) from Gelman and were ready for the injection into the HPLC system.

RESULTS

The catecholamines were quantitated using the internal standard calculation based on peak height. The peak identified on the retention time is related to the DHBA peak. Sample concentrations were quantitated by comparing the peak height ratio obtained for the sample to the ratio obtained for the extracted calibration standard (E, NE, DHBA : 1 ug/mL each).

The within-run and the between-run precision results are presented in the tables 1 and 2.

TABLE 1

Within-run Precision

	NE	E
n	17	17
X	151.13	26.71
S.D	5.80	1.12
C.V.	3.84	4.22

TABLE 2

Between-Run Precision.

	NE	E
n	10	10
X	149.44	28.80
S.D	8.33	1.80
C.V	5.58	6.27

Lyphocheck level II (Lot No 11200) mean values with the BIO-RAD extraction columns and the HPLC test were respectively 175 ug/L (151-199) and 31 ug/L (25-37) for NE and E.

The analytical recovery on the columns for norepinephrine and epinephrine were determined by comparing the peak height ratios of the extracted compounds vs DHBA added after the elution with the unextracted standards.

The results for NE and E were 80.33 % and 80.89 % after extraction on weak cation exchange (WCE) column and respectively 61 % and 60 % with the extraction on WCE and alumina adsorption. The method was linear up to 400 ug/24 h.

The degree of interference varies from sample to sample. To determine the specificity of the WCE-alumina method, we added potential interfering substances to a normal urine as : normetanephrine, metanephrine, VMA, HVA, 5-HIAA, MHPG, 3.4 dihydroxyphenyl glycol, 3.4 dihydroxymandelic acid, 3.4 dihydroxyphenylacetic acid, p-hydroxybenzoic acid.

Antihypertensive drugs as alpha-methyldopa which was metabolised with similar biogenic amine form could be isographic with the dopamine peak. Dilution of the urinary sample with 0.05M HCL before treatment solve this problem. No problem has been observed with L-dopa.

DISCUSSION

The method developped by Riggin and Kissinger (7,8,9) based on the combination of the ion exchange resin and the alumina adsorption constitues at this time the higher analytical performance for the preparation of the catecholamines in urine. Coupling weak cation exchange resin (WCE) and alumina provides the most sensitive and specific chromatograms (Figures 2,3) in comparison with faster treatments as WCE or alumina only.

Chemically bonded materials as Bond-Elut from Analytichem, recently introduced on the market for the analysis of catecholamines seems to be a compromise between the high analytical performance of WEC-alumina and the rapidity of the WCE and alumina procedures (11,14,15).

BIO-REX 70 is a weakly acidic cation exchanger. The resin was regenerated by a batch method, converted to its appropriate sodium form and equilibrated in 0.1M phosphate buffer at pH 6,5.

The functional group is carboxylic acid. It is a non spherical copolymer of methacrylic acid and divinylbenzene with macropores which average a diameter of 200 angstroms (figure 4). When hydrated, the ion exchange resin is most fully swollen. The swelling decreases when the ionic strength of the solvent is increased as well as non polar solvent are added. Resin size was also modified in the range of 100 % with the conversion from the hydrogen to the sodium form.

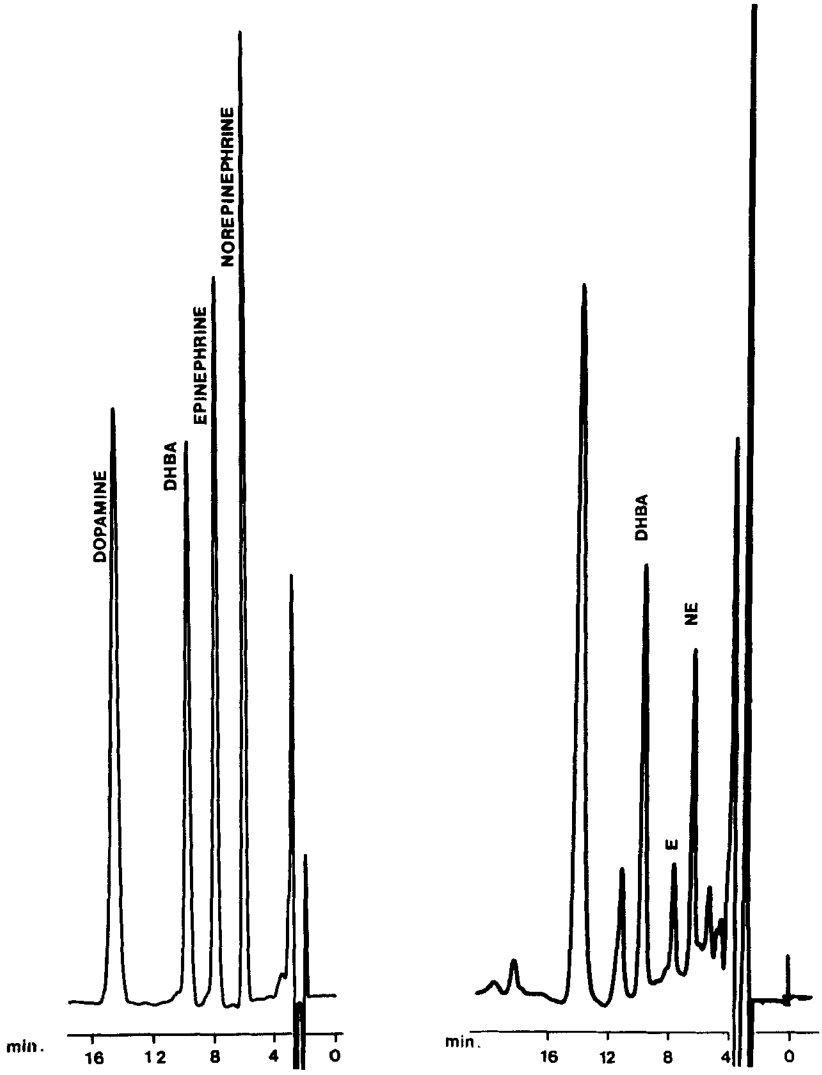

FIGURE 2.

Left : chromatogram of an aqueous solution containing 5 ng of norepinephrine, epinephrine and DHBA after purification by WCE-alumina.
Right : chromatogram of an urine sample containing 84 ug/24H of norepinephrine and 25.16 ug/24H of epinephrine. Common conditions : detector 0.40V, sensitivity gain 10 x 20, flow-rate 0.60 ml/min.

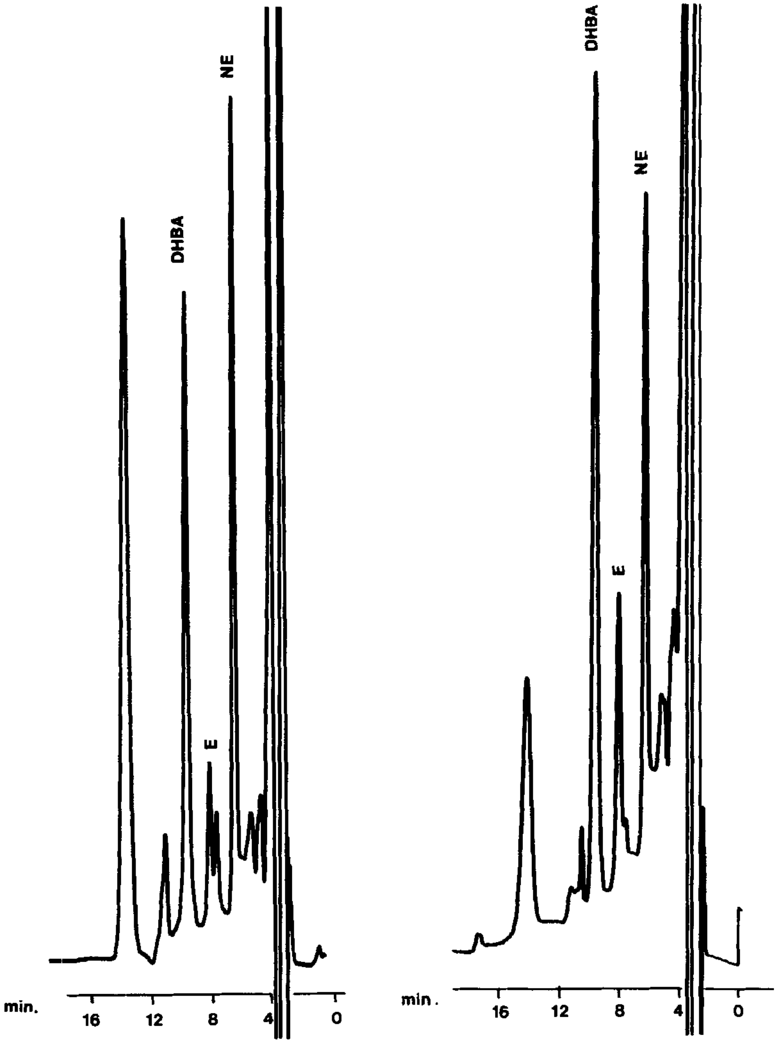

FIGURE 3.

Left : chromatogram of lyphocheck urine control (level II) containing 151,13 ug/L of NE and 26.71 ug/L of E.
Right : chromatogram of Biotrol urine control (lot A 02260) containing 93.78 ug/L of NE and 34.9 ug/L of E. Same conditions for both chromatograms : detector 0.40V, sensitivity gain 10 x 40, flow-rate 0.60 ml/min.

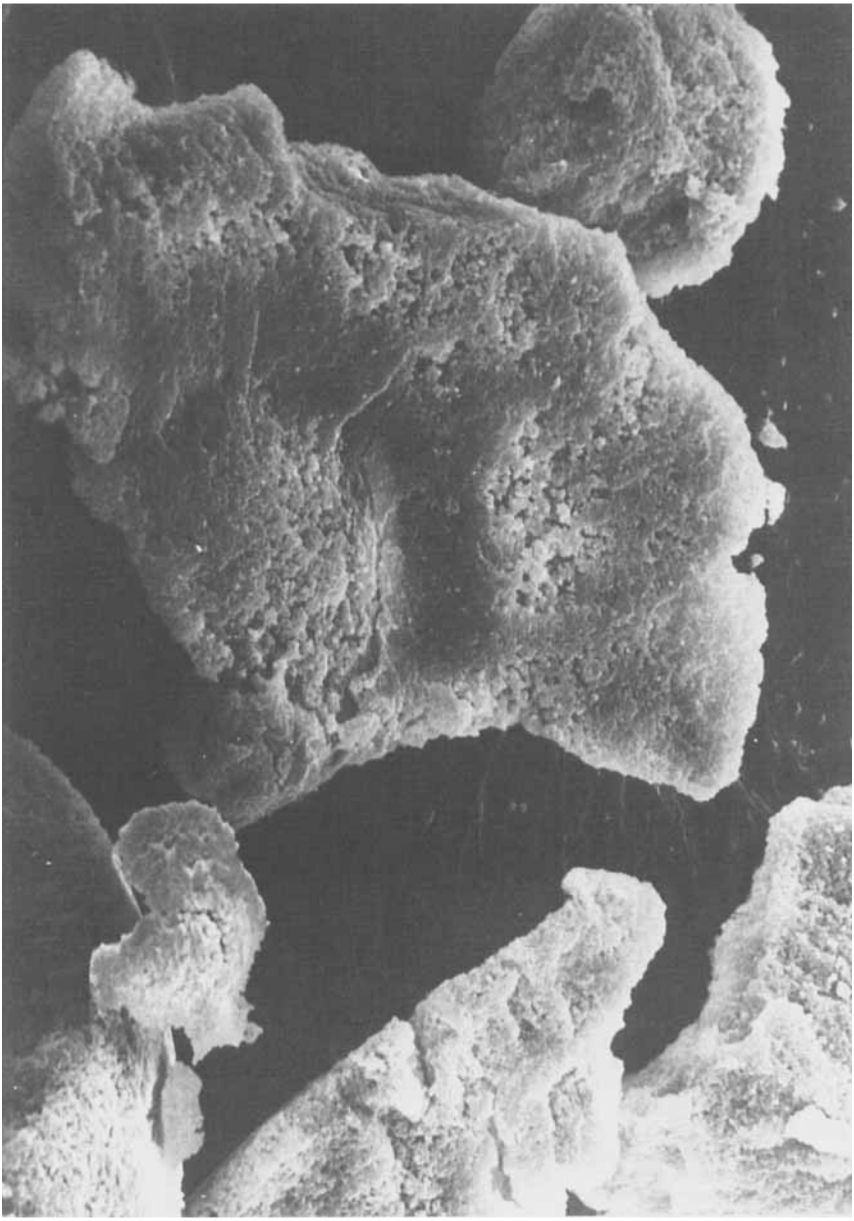

FIGURE 4.

Scanning electron micrograph (150 x) showing the amorphous aspect of the Bio-Rex 70 50-100 mesh.

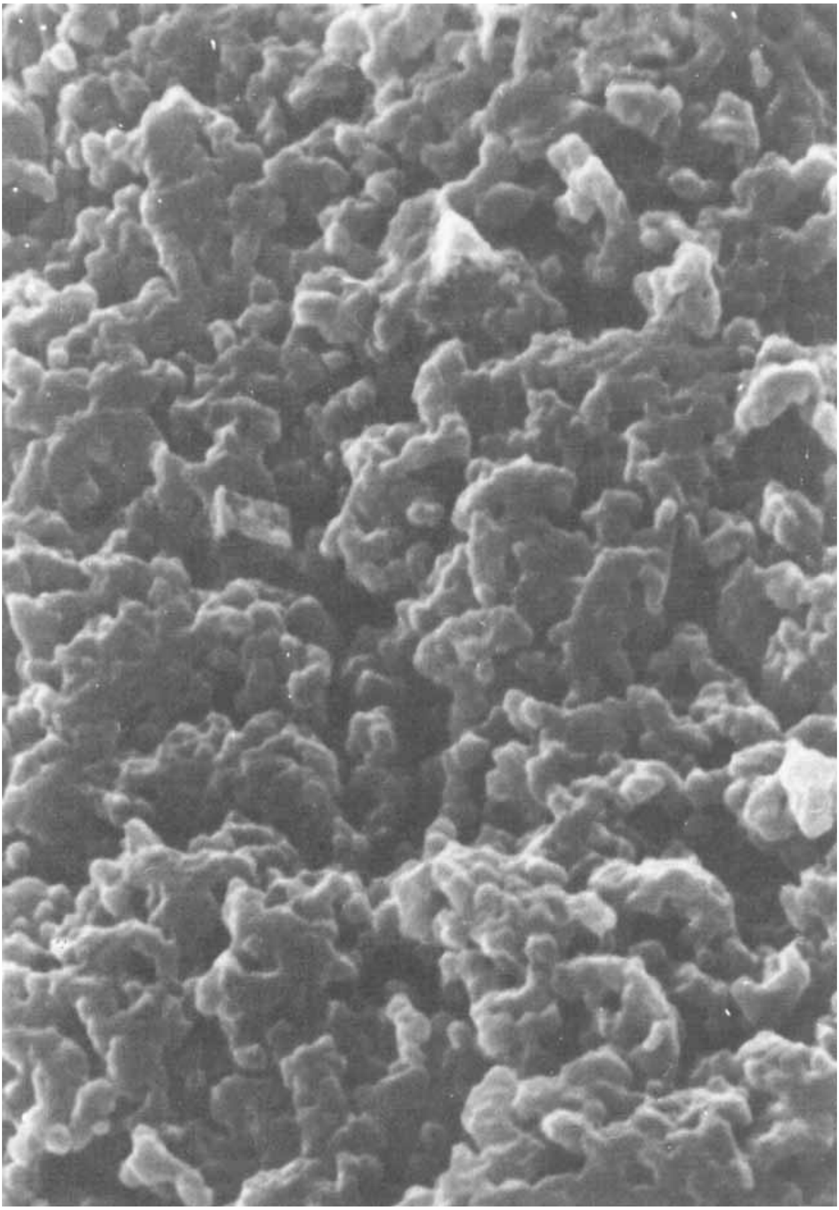

FIGURE 5.

Electron micrograph (11 250 x) of the Bio-Rex 70 micropores which vary in size with pH and ionic strength.

Because it is non spherical, BIO-REX 70 tended to pack irregularly. Moreover, during re-equilibration the particules swell, resulting in a more close packing increasing the resistance to the flow (Figure 5). In these conditions, the resin began to collapse causing some decrease in the macropore size and variability in the separation characteristics. It was very important to safely control the rotation speed in order to prevent excessive clogging of the chromatographic media, keeping it wet enough to conduct the analysis in good analytical conditions. Incorrect equilibration or/and poor hydratation underyield unreproductible results.

The approximate time required for the "clean-up" of 8 samples was 140 minutes for the BIO-RAD method and less than 50 minutes for the method described. This allows a significant increase of the HPLC time for other analysis such as : drugs monitoring... Reproducible and time saving, this inexpensive procedure eliminates problems such as front extraction, back extraction, emulsion, evaporation, large solvent volume and glass consumption. Effective and dedicated for clinical chemistry developments, its applications'field can be enlarged to : toxicology, food and pharmaceutical quality analysis.

AKNOWLEDGMENTS

We thank Dr. Marchal and Dr. Mrena for their contribution to the realisation of the electron micrograph pictures.

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