ION-EXCHANGE RESIN PAPER FOR SEPARATION OF THE ACIDIC URINARY METABOLITES OF NOREPINEPHRINE-2-^{14}C IN HUMAN SUBJECTS*

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(Received September 30th, 1963)

In the course of studies on the metabolism of labeled norepinephrine in essential hypertensive patients, a method has been developed for the separation of the acidic metabolites using ion-exchange paper, which was thought worthwhile to report here, in addition to preliminary results obtained in normotensive and hypertensive individuals.

GOODALL *et al.*[1], KIRSHNER *et al.*[2,3] and MASUOKA *et al.*[4] have described methods for separating and determining the metabolites of norepinephrine-^{14}C by column chromatography, using CG-50 and Dowex-1 ion-exchange resins and alumina columns.

The method reported here is essentially an adaption from KIRSHNER *et al.* and GOODALL *et al.*[1-3] but substituting Dowex-1 ion-exchange resin-impregnated paper for Dowex-1 columns. Since ion-exchange resin-loaded papers have become available, several reports have appeared in the literature, showing successful application of these papers in the separation of a wide variety of organic and inorganic compounds (see Reeve-Angel brochures for literature, 9 Bridewell Place, Clifton, N.J.).

METHOD AND RESULTS

DL-Norepinephrine-2-^{14}C-acetate (purchased from the Commissariat à L'Énergie Atomique, Gif-sur-Yvette, Seine-et-Oise, France, who synthesized it by a method developed in this laboratory by HOWTON, MEAD AND CLARK[5]) 20 mC/mmol, 46 μC (370 μg, total) was sterilized by the millipore membrane filter technique and infused intravenously in 500 ml 5% dextrose over a 30 min period (equivalent to 0.09 μg/kg/min L-norepinephrine), into 6 normotensives and 6 frank essential hypertensives without abnormal renal function. The urines were collected in containers with acetic acid at hourly intervals from the start of infusion for 6 h, then every 6 h for 24 h. After removing an aliquot of the urine specimen for determining total radioactivity relative to that infused, a 5 ml aliquot was adjusted to pH 6.1 with NH_4OH and passed through a 3 × 1 cm column of Amberlite CG-50 resin, Type 2, 200–400 mesh (Rohm and Haas, Co., Philadelphia), in the NH_4^+ form at pH 6.1 at the rate of 4–5 drops/min.

* Supported in part by U.S. Public Health Service (MY-3663:H-5329), Los Angeles County Heart Association (225), American Heart Association (60-G-27), and National Association Mental Health.

The resin was previously cycled and converted to the NH_4^+ form by the method of HIRS, MOORE AND STEIN[6], *cf.* KIRSHNER AND GOODALL[3]. The column was washed with 15 ml H_2O and after removing an aliquot from the combined effluent and wash for estimating the radioactivity of the unretained neutral and acidic metabolites, the effluent plus wash was lyophilized nearly to dryness. The column, retaining norepinephrine and normetanephrine, was eluted with 20 ml 0.5 *M* acetic acid, made to 25 ml, and 0.1 ml aliquots counted in a Packard Tri-Carb liquid scintillation spectrometer in 10 ml of phosphor consisting of 30% absolute ethanol and 70% toluene containing 0.4% of 2,5-diphenyloxazole (PPO) and 0.01% (1,4-bis-2-[5-phenyloxazolyl]-benzene) (POPOP).

The results were expressed in disintegrations per minute by use of an internal standard. The lyophilized CG-50 effluent plus wash, containing the acidic metabolites, was made to 0.5 ml volume with H_2O and a 10–50 μl aliquot spotted in six steps, drying with nitrogen at each step, along with 3-methoxy-4-hydroxymandelic (MOMA) and 3,4-dihydroxymandelic acid (DOMA) standards, on 18 × 22 in. sheets of Reeve-Angel Type SB-2 (Dowex-1 or IRA-400) ion-exchange resin paper (Reeve-Angel & & Co., Inc., Clifton, N. J.) in the acetate form. To avoid streaking it is necessary to convert it to this form from the chloride form in which it is supplied. This was done by allowing 4 *M* ammonium acetate–acetic acid, pH 6.5, to wash through the paper by descending development in a Chromatocab for several days or until the buffer dripping off the bottom of the paper showed only slight cloudiness with $AgNO_3$. The wet paper then was placed in a large enameled pan, washed twice with distilled H_2O and dried. The acid metabolites and standards, as many as 12 per sheet, were developed with 10.0 *M* ammonium acetate, pH 5.0 by the descending technique in a Chromatocab at constant temperature (25°) for approximately 26 h, or until the solvent front was 39–40 cm from the origin.

It was found that development with 10 *M* gave the best separation of standards and least streaking, with the R_F values of DOMA and MOMA being 0.35 and 0.53 respectively; in 4 *M* they were 0.13 and 0.29; in 1 *M*, 0.04 and 0.07 respectively. The paper was dried overnight in air, and the spots, located readily under ultraviolet light, marked and a tracing of the chromatogram made. The paper then was cut into 2 cm wide strips which were taped end-to-end and scanned in a Vanguard strip-scanner (Vanguard Instrument Co., La Grange, Ill.) to locate the radioactive peaks. For quantitative estimation of activity, the 2 cm longitudinal strips were cut into 1 cm wide segments and counted directly in the liquid scintillation spectrometer in the toluene phosphor without ethanol. No radioactivity eluted off, nor were there problems of orientation of the paper toward the phototube, as reported by WANG AND JONES[7] and LOFTFIELD AND EIGNER[8], because the papers are small enough to fit into the counting vials without folding, and the entire paper size showed no relationship to counting rate, which confirms the findings of BOUSQUET AND CHRISTIAN[9]. Approximately 40% counting efficiency was obtained, using an internal standard.

The results obtained by use of the resin paper compare favorably with the results from a Dowex-1 × 2 resin column, as shown in Figs. 1 and 2. These results were obtained from a urine specimen obtained 1 h after the infusion of norepinephrine-2-^{14}C, as described above, into a normotensive individual. Note that the solvent front is equivalent to the initial fractions from the column. The peaks from the column have been partially identified by GOODALL *et al.*[1]; *cf.* KIRSHNER *et al.*[2], the first as nor-

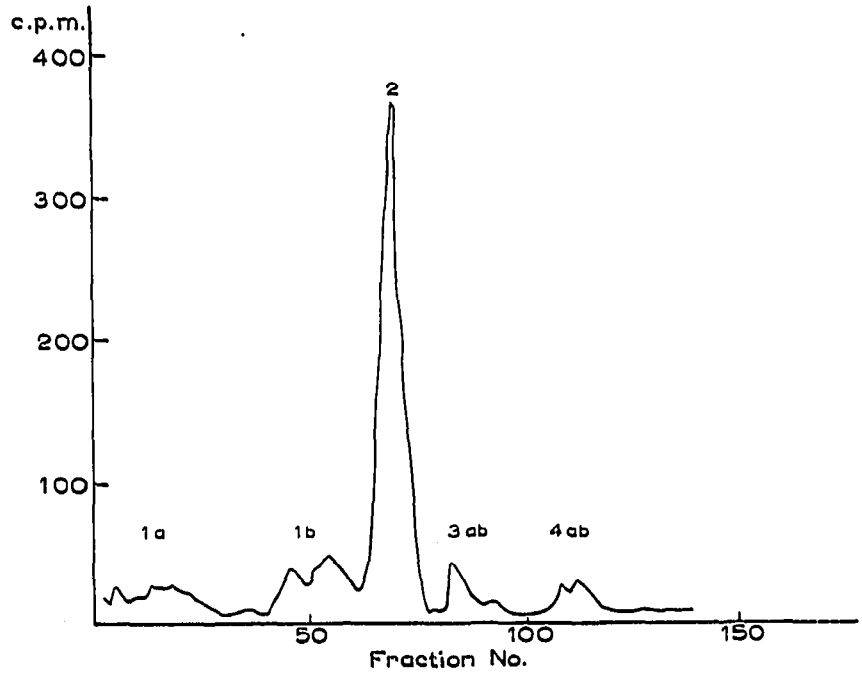

Fig. 1. Separation of metabolites of norepinephrine-2-^{14}C on an ion-exchange column. 1a = normetanephrine conjugate; 1b = unknown; 2 = MOMA; 3a, 3b = DOMA; 4a, 4b = unidentified.

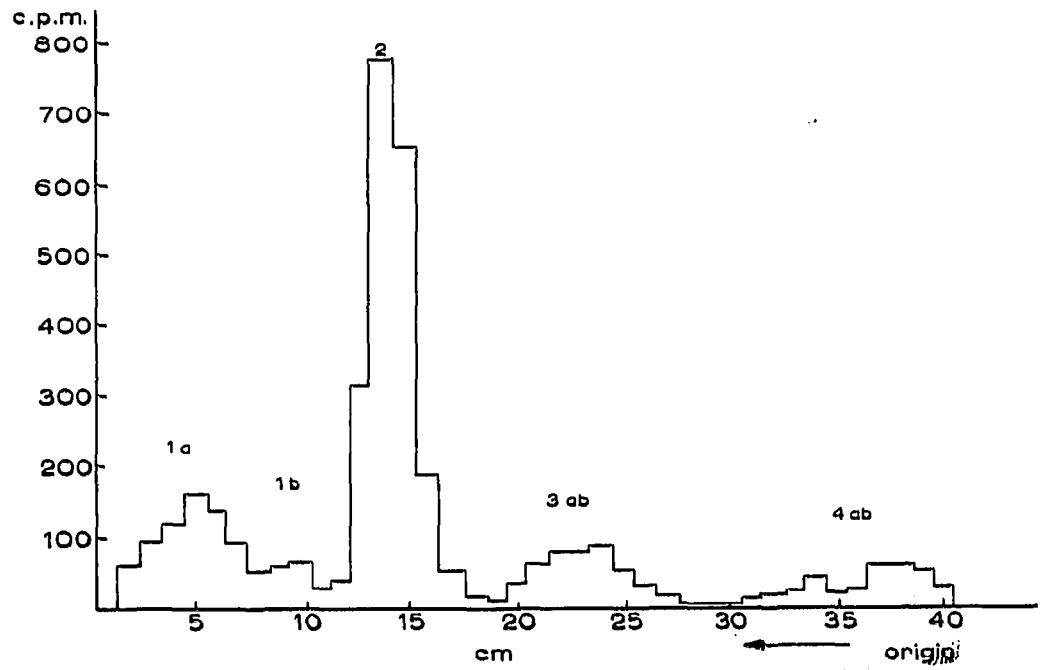

Fig. 2. Separation of metabolites of norepinephrine-2-^{14}C on ion-exchange paper. 1a = normetanephrine conjugate; 1b = unknown; 2 = MOMA; 3a, 3b = DOMA; 4a, 4b = unidentified.

metanephrine conjugate, followed by a small unknown peak (1b), the largest peak (2) as MOMA, followed by DOMA (3a + 3b) and several small unidentified peaks (4a + 4b). The percentage of radioactivity under each peak from the column and the ion-exchange paper are compared in Table I.

TABLE I

PERCENTAGE RADIOACTIVITY UNDER EACH PEAK

Peak No.	*1a and 1b*	*2*	*3a and 3b*	*4a and 4b*	*Total (%)*
Column	27.2	54	7	6.7	94.9
Paper	21.5	55	11	7	94.5

The advantages of the ion-exchange paper over the column method are: shorter time, elimination of fraction collection, and possibility of running up to 12 samples simultaneously.

There was no significant difference between the hypertensives and normotensives in the labeled peaks found, whether compared on the basis of urinary creatinine, for each time of collection, or totalled. This is in agreement with the reports of SJOERDSMA[10], SATO *et al.*[11], GITLOW, *et al.*[12] and others, that apparently there is no defect in the catabolism of *exogenously* administered norepinephrine in hypertension. However, before it can be concluded unequivocally that a defect in the catabolism of endogenous catecholamine may be causally related to the etiology of essential metabolism, a careful study must be made of all endogenous metabolites present. This has as yet not been accomplished, although the elegant studies of LABROSSE *et al.*[13] have accounted for most of the major ones from exogenously administered ^{3}H-labeled material.

SUMMARY

The acidic urinary metabolites of norepinephrine-2-^{14}C were rapidly and conveniently separated and quantitatively determined by first removing the amines on a CG-50 column, which can be eluted and measured. The unretained normetanephrine conjugate, 3-methoxy-4-hydroxymandelic acid, 3,4-dihydroxymandelic acid and several unknowns are separated on Dowex-1 ion-exchange resin-impregnated paper by developing in the descending direction with 10 *M* ammonium acetate. After scanning to locate the areas, the radioactive areas are cut out and counted directly in the liquid scintillation counter.

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