CHROM. 5857

SEPARATION OF NOREPINEPHRINE AND ITS FIVE MAJOR METABOLITES BY PAPER CHROMATOGRAPHY*

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SUMMARY

Over one-hundred solvent systems were tested on Whatman No. 1 paper and over fifty solvent systems were tested on silica gel loaded paper, cellulose phosphate paper, and carboxymethyl cellulose paper to find a method for separating norepinephrine and its five major metabolites. Although no single solvent system adequately separated all six compounds on any of the papers used, a number of solvents did separate the compounds into chemically related groups. Complete separation of all six compounds was obtained by chromatography in two different solvent systems on cellulose phosphate paper. *n*-Butanol–95% ethanol–water (1:1:1) separated norepinephrine from normetanephrine with virtually no interference from the deaminated metabolites. *n*-Butanol–pyridine–water (14:3:3) retained the amines at the origin and provided complete separation of the deaminated metabolites along the remainder of the paper. Very small amounts of sodium chloride increased the mobility of the amines on the cation-exchange papers.

INTRODUCTION

Before initiating a long-term study of norepinephrine (NE) metabolism in vascular tissue, an attempt was made to find a suitable method for the routine assay of tritiated norepinephrine (^{3}H-NE) and its five major metabolites: normetanephrine (NM), 3,4-dihydroxyphenylethylglycol (DP), 3,4-dihydroxymandelic acid (DM), 3-methoxy-4-hydroxymandelic acid (VM), and 3-methoxy-4-hydroxyphenylethylglycol (MP). All of the column chromatographic methods available are quite laborious and the most commonly used method[1] does not separate all five of these metabolites from one another. The present investigation was undertaken to find a paper chromatographic (PC) system which might serve as the basis for a simpler, more reliable and less tedious assay. A suitable PC system must completely separate NE and the above metabolites from one another. Although several PC systems have previously been used for the separation of catecholamines and their metabolites, the R_F values for NE and all five of its major metabolites were only reported for two of these systems[2]. To find the best PC system, the author has determined the R_F

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values for NE and all five of the above metabolites in a number of solvent systems which have been used with catecholamines. Four special types of chromatography paper have been used in addition to Whatman No. 1 paper.

METHODS

Norepinephrine and its major metabolites were dissolved in methanol and stored at —10°. 10 to 25 μg of the compounds were spotted separately on the chromatography paper in 5-μl aliquots and the spots were dried at room temperature. The chromatograms were equilibrated with the solvent system for at least 6 h and developed by descending chromatography for at least 20 cm. When a two-phase solvent system was used, the chromatograms were equilibrated with the aqueous phase and developed with the organic phase. All chromatograms developed with a solvent system containing phenol were washed by passing them back and forth several times through two portions of distilled benzene as soon as they were taken out of the chromatography tank.

The spots were usually detected by spraying the chromatograms with diazotized sulfanilic acid. The spray was prepared by mixing one part of 5 % sodium nitrite solution with two parts of 0.9 % sulfanilic acid in 1 *N* HCl. After chilling on ice for 15 min, the mixture was added to three parts of cold 20 % sodium carbonate solution and the spray was used immediately[3]. All three catechols were stained light tan. The three non-catechols were stained deep orange. Chromatograms developed with a solvent containing phenol were stained according to the methods described by LANGER[2].

Some chromatographic systems were tested to determine whether they produced any cross-contamination of the spots. For this purpose, 20 μg of NE and the metabolites were spotted on the paper along with 0.1 μCi ^{3}H-NE, ^{3}H-NM, or ^{3}H-VM. The chromatograms were equilibrated, developed, and stained as described above. Each chromatogram was then cut into several consecutive segments according to the location of the spots on the chromatogram. Each segment contained a spot or the area between two spots. The segments were placed into individual nylon liquid scintillation counting vials. The radioactivity was eluted from the paper by adding 1 ml of 0.2 *N* HCl to each vial and shaking the vials for 30 min. 10 ml of counting solution were added directly to each vial and the samples were counted in a liquid scintillation counter. The counting solution contained 5g of 2,5-diphenyloxazole, 0.5 g of 1,4-bis-2-(4-methyl-5-phenyloxazolyl)-benzene and 167 ml (180.2 g) Beckman Biosolv BBS 3 per liter of toluene. The net c.p.m. in each segment was then expressed as a percent of the total net c.p.m. on the chromatogram.

Norepinephrine and the five metabolites were purchased from Sigma Chemical Co. The solvents were obtained from Fisher Scientific Co. (Certified Grade) or Matheson, Coleman and Bell (Reagent Grade). Acid-washed Whatman No. 1 chromatography paper was prepared by over-developing Whatman No. 1 paper with 0.01 *N* HCl. Several milliliters of acid were permitted to run off the end of the paper. The paper was air-dried at room temperature and used the same day. Silica gel loaded paper, cellulose phosphate paper, and carboxymethyl cellulose paper were obtained from Reeve Angel. Tritiated NE, NM, and VM were purchased from New England Nuclear Co. The ^{3}H-NM and ^{3}H-VM were purified by chromatography on cellulose

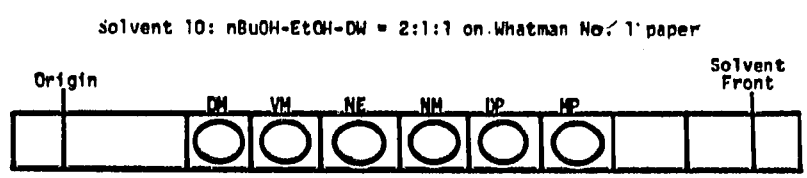
phosphate paper. Solvent 9 was used for ^{3}H-NM and solvent 20 was used for ^{3}H-VM (see Table I). The unstained chromatograms were analyzed in a radiochromatogram scanner to localize the radioactive peak with the same R_F value as authentic NM or VM. This portion was cut out of the chromatogram and the radioactivity was eluted with 0.1 *N* acetic acid containing 10 μg of disodium ethylenediamine tetraacetate/ml and 1 mg of ascorbic acid/ml. The samples of ^{3}H-NE obtained from New England Nuclear have not needed further purification but were diluted with the same acetic acid eluting fluid mentioned above. All three tritiated compounds were stored at 5° in aliquots sealed under nitrogen.

RESULTS

Whatman No. 1 paper

Table I lists the R_F values of NE and its five most common metabolites in 42 solvent systems on Whatman No. 1 paper. All of these solvent systems except two have been used previously for the chromatography of catecholamines and other phenols, but not all of them were originally used with Whatman No. 1 paper. To determine whether any of these solvent systems adequately separated all six compounds from one another, the separation index was calculated for each solvent system by taking the difference between the R_F values of the two spots which were closest together on the chromatogram. All other things being equal, an increase in the separation index indicates that a better separation of all six compounds has been obtained. Solvent 10 consistently provided the largest separation index without any apparent streaking of the spots. Even with this solvent system there was usually some overlap of the NE and VM spots. When ^{3}H-NE, ^{3}H-NM, and ^{3}H-VM were applied to separate chromatograms and developed in this solvent system, significant amounts of each compound were found in spots of some of the other compounds (Fig. 1). This was especially true of the amines. Both ^{3}H-NE and ^{3}H-NM were streaked back towards the origin. Thus, extensive cross-contamination between the spots makes this solvent system unsatisfactory for use in an assay of NE and its metabolites.

Although the solvent systems listed in Table I did not adequately separate all six compounds, several systems did separate the compounds into chemically related groups. Solvents 19, 20, 21, 33 and 34 separated the compounds into three groups: the two amines (NE and NM), the two glycols (DP and MP), and the two



	n	1	2	3	4	5	6	7	8	9
Spot ^{3}H-NE	6	2.2 ±0.5	1.9 ±0.3	29.2 ±4.1	65.0 ±3.7	1.3 ±0.3	0.3 ±0.1	0.1 ±0.03	0.1 ±0.02	0.01 ±0.01
Spot ^{3}H-NM	6	2.6 ±0.6	2.8 ±0.7	5.2 ±1.4	6.4 ±1.7	80.7 ±4.9	1.5 ±0.2	0.5 ±0.1	0.4 ±0.1	0.03 ±0.01
Spot ^{3}H-VM	6	3.2 ±0.9	2.1 ±0.4	67.0 ±3.7	18.4 ±3.5	0.6 ±0.1	0.5 ±0.1	0.5 ±0.1	7.3 ±1.4	0.6 ±0.2

Fig. 1. Cross-contamination of spots on Whatman No. 1 paper with solvent 10. Values are net c.p.m. in each segment expressed as a percent of total net c.p.m. on chromatogram; mean ± S.E.

TABLE I

$R_F \times 100$ VALUES OF NOREPINEPHRINE AND ITS MAJOR METABOLITES ON WHATMAN NO. 1 PAPER

Abbreviations used in the list of solvents are as follows: AcO = Acetone; *n*AmOH = *n*-amyl alcohol; *t*AmOH = *tert.*-amyl alcohol; *n*BuOH = *n*-butanol; *sec.*BuOH = sec.-butanol; *t*BuOH = *tert.*-butanol; BZ = benzene; Diox = dioxane; DW = distilled water; EtAc = ethyl acetate; EtOH = ethanol; HAc = acetic acid; HFor = formic acid; HPro = propionic acid; MeOH = methanol; NH_3 = ammonium hydroxide (28% NH_3); 5% NH_3 = ammonium hydroxide diluted to 5% NH_3; PhOH = phenol crystals; liq PhOH = liquid phenol; *i*PrOH = isopropanol; Pyr = pyridine; Tol = toluene.

No.	*Solvent*	*$R_F \times 100$ value*						*Separation index*[a]	*Solvent system previously used by (ref.)*[b]
		NE	*NM*	*DP*	*DM*	*MP*	*VM*		
1	MeOH–*n*BuOH–BZ–DW (4:3:2:1)[c]	36	53	60	16	74	27[d]	7	5
2	MeOH–*n*BuOH–BZ–DW (4:4:4:1)	36	50	59	13	74	26[d]	9	5
3	MeOH–*n*BuOH–BZ–DW (2:1:1:1)	64	72	73	50	83	61	1	5
4	MeOH–BZ–*n*AmOH–2 *N* HCl (74:70:35:25)	33	50	80	73	79	79	0	5
5	MeOH–*n*BuOH–BZ–Pyr–DW (2:1:1:1:1)	65	76	79	44	83	57	3	5
6	*i*PrOH–5% NH_3 (4:1)[c]	0[e]	61	0[e]	20	78	38	0	6
7	*i*PrOH–NH_3–DW (8:1:1)	0[e]	49	0[e]	0[e]	67	30	0	7
8	*i*PrOH–0.2 *M* NH_4Ac (1:2)[i]	72	76	75	71	84	79	1	8
9	*n*BuOH–EtOH–DW (1:1:1)	58	65	65	59	71	67	0	
10	*n*BuOH–EtOH–DW (2:1:1)[f]	43	54	64	23	75	32	9	5
11	*n*BuOH–EtOH–DW (4:1:1)	24	36	56	9	67	17	7	9
12	*n*BuOH–EtOH–BZ–DW (8:5:4:4)[c,g,i]	32	49	63	21	75	31	1	9
13	*n*BuOH–EtOH–1 *N* HAc (35:10:10)[i]	33	46	59	38	71	50	5	10
14	*n*BuOH–EtOH–HAc–DW (2:1:1:1)[c]	50	62	57	54	68	66	2	
15	*n*BuOH–HAc–DW (4:1:1)	32	50	55	54	71	71	0	7
16	*n*BuOH–HAc–DW (12:3:5)	40	54	57	55	73	71	1	11
17	*n*BuOH–HAc–DW (5:1:3)[f,g]	43	56	64	61	77	75	2	12
18	*n*BuOH saturated with 1 *N* HCl[f,g]	20	33	57	54	74	54	0	5
19	*n*BuOH–Pyr–DW (1:1:1)	67	70	83	52	83	57	0	5
20	*n*BuOH–Pyr–DW (14:3:3)	24	33	63	8	73	14	6	11
21	*n*BuOH–Pyr–Diox–DW (14:4:1:1)[f]	21	24	66	5	72	8	3	13

22	*n*BuOH–Pyr–0.2 *N* NaAc (1:1:1)	53	61[d]	81	41	83	46	2	5
23	*n*BuOH–Pyr (1:1) saturated with 1 *N* NaAc[g]	59	63	81	46	84	52	3	5
24	*sec.*BuOH–pH 3.9 buffer (4:1) (pH 3.9 buffer; Pyr–HAc–DW (10:41:100))	13	22	51	29	65	45	6	14
25	*t*BuOH–AcO–HFor–DW (160:160:1:39)	43	51	72	75[d]	79	80	1	5
26	*t*BuOH–AcO–HPro–DW (160:160:1:39)	41	47	71	28[d]	79	35	6	5
27	*t*AmOH–NH_3 (4:1)	0[e]	27	0[e]	0[e]	47	3	0	14
28	EtAc–HAc–DW (10:3:6)[g]	78	84	77	78	84	84	0	12
29	BZ–HAc–DW (125:72:3)[c]	15[d]	55[d]	29	17	67	56	0	11
30	BZ–HPro–DW (4:1:1)[g]	60[d]	78	60	52	77	70	0	15
31	BZ–HPro–DW (2:2:1)[c,g]	36[d]	58[d]	43[d]	31[d]	63[d]	51[d]	5	13
32	BZ–HPro–DW (2:1:1)	30	44	32	29	48	44	0	5
33	Tol–EtAc–Pyr–MeOH–DW (1:1:1:1:1)	53	61	77	15	79	29	2	5
34	Tol–EtAc–MeOH–0.1 *N* HCl (1:1:1:1)	64	69	80	48	80	47	0	5
35	Tol–HAc–EtAc–DW (16:8:4:1)[c,i]	5	15	23	15	56	47	0	16
36	PhOH–0.02 *N* HCl (4:1)	53	80	62	28	?[h]	?[h]	?	17
37	PhOH–0.1 *N* HCl (85:15)[f,i]	41	82	57	17	86	51	4	2
38	Solvent 37–*n*BuOH–HAc (2:2:1)[f]	21	58	67	35	?[h]	?[h]	?	2
39	Liq PhOH in an atmosphere of 1 *N* HCl	47	79	64	41	?[h]	?[h]	?	18
40	Anisole–HAc–DW(70:27:3)	1	4	6	3	29	22	1	19
41	$CHCl_3$–HAc–DW (13:6:1)[c]	9	49	24	15	66	49	0	20
42	$CHCl_3$–HAc–DW (2:1:1)	58[d]	84	67	61	82	77	2	5

[a] The separation index is the difference between the R_F values of the two spots which are closest together on the chromatogram.

[b] Although these solvent systems have been used to separate catecholamines and their metabolites only one of the authors reported the R_F values for all five of the most common metabolites of norepinephrine.

[c] Values obtained with this solvent system were not reproducible.

[d] This spot was streaked.

[e] This compound is probably unstable in this solvent system. An abnormally colored spot was observed at the origin with considerable streaking toward the solvent front.

[f] Values obtained with this solvent system were quite reproducible.

[g] This was a two-phase solvent system. The papers were equilibrated with the aqueous phase and developed with the organic phase.

[h] No spot was observed for this compound when the chromatogram was stained. The compound was probably washed from the paper by the benzene used to remove the phenol.

[i] Solvent originally described for use on a support media other than Whatman No. 1 paper.

acids (DM and VM). Solvents 29, 30, 32, 41 and 42 separated the compounds into two groups: the catechols (NE, DP, and DM) and the non-catechols (NM, MP, and VM).

Several attempts were made to improve the separation obtained on Whatman No. 1 paper by changing the polarity of several solvent systems listed in Table I and by the addition of acetic acid, pyridine, or piperidine to several solvents. In addition, several solvent systems were tested on acid-washed Whatman No. 1 paper and on silica gel loaded paper. None of these chromatographic procedures provided a satisfactory separation of NE and the five metabolites tested here. The results of these additional studies will be supplied on specific request to the author.

Cellulose phosphate paper

Cation-exchange papers were tested in an attempt to overcome the cross-contamination of the spots caused by streaking of the amines back toward the origin.

TABLE II

$R_F \times 100$ VALUES OF NOREPINEPHRINE AND ITS MAJOR METABOLITES ON CELLULOSE PHOSPHATE PAPER

For abbreviations, see Table I.

No.	*Solvent*	*$R_F \times 100$ value*						*Separation index*[a]
		NE	*NM*	*DP*	*DM*	*MP*	*VM*	
1	MeOH–*n*BuOH–BZ–DW (4:3:2:1)	1	6	66	60	80	79	1
6	*i*PrOH–5% NH_3 (4:1)	0[e]	57	0[e]	0[e]	77	55	0
7	*i*PrOH–NH_3–DW (8:1:1)	0[e]	59	0[e]	0[e]	74	44	0
8	*i*PrOH–0.2 *M* NH_4Ac (1:2)	18	36	77	77	84	84	0
9	*n*BuOH–EtOH–DW (1:1:1)[f]	5	33	73	69	83	80	3
10	*n*BuOH–EtOH–DW (2:1:1)	4	15	67	62	81	77	4
12	*n*BuOH–EtOH–BZ–DW (8:5:4:4)[g]	4	12	69	60	83	77	6
13	*n*BuOH–EtOH–1 *N* HAc (35:10:10)	2	7	58	49	75	68	5
14	*n*BuOH–EtOH–HAc–DW (2:1:1:1)[f]	8	34	62	55	79	74	5
15	*n*BuOH–HAc–DW (4:1:1)	5	23	56	48	78	73	5
16	*n*BuOH–HAc–DW (12:3:5)	10	33	63	56	81	78	3
18	*n*BuOH saturated with 1 *N* HCl	14	18	60	54[d]	79	76	3
19	*n*BuOH–Pyr–DW (1:1:1)	14	31	91	73	94	80	3
20	*n*BuOH–Pyr–DW (14:3:3)[f]	0.3	2	67	31	81	48	1.7
21	*n*BuOH–Pyr–Diox–DW (14:4:1:1)[f]	1	3	73	45	81	62	2
22	*n*BuOH–Pyr–0.2 *N* NaAc (1:1:1)	9	19	88	64	90	74	2
23	*n*BuOH–Pyr (1:1) saturated with 1 *N* NaAc	20	30	83	60	89	70	6
24	*sec*.BuOH–pH 3.9 buffer (4:1) (pH 3.9 buffer; Pyr–HAc–DW (10:41:100))							
27	*t*AmOH–NH_3 (4:1)	0[e]	41	0[e]	0[e]	62	5	0
29	BZ–HAc–DW (125:72:3)[c]	3	31[d]	16	5	69	47[d]	2
31	BZ–HPro–DW (2:2:1)[c,g]	6	48[d]	49	30	73	59	1
32	BZ–HPro–DW (2:1:1)[g]	5	30[d]	39	24	53[d]	49[d]	4
35	Tol–HAc–EtAc–DW (16:8:4:1)	1	4	8	3	39	23	1
36	PhOH–0.02 *N* HCl (4:1)	5	42	63	34	?[h]	?[h]	?
37	PhOH–0.1 *N* HCl (85:15)[f]	3	35	59	26	?[h]	?[h]	?
38	Solvent 37–*n*BuOH–HAc (2:2:1)[f]	6	51	43	19	?[h]	?[h]	?
39	Liq. PhOH in an atmosphere of 1 *N* HCl	5	51	64	34	?[h]	?[h]	?
40	Anisole–HAc–DW (70:27:3)	0	4	7	3	37	22	1
41	$CHCl_3$–HAc–DW (13:6:1)	1	16	12	4	60	37	3
42	$CHCl_3$–HAc–DW (2:1:1)	16[d]	68[d]	67	56	82	77	1

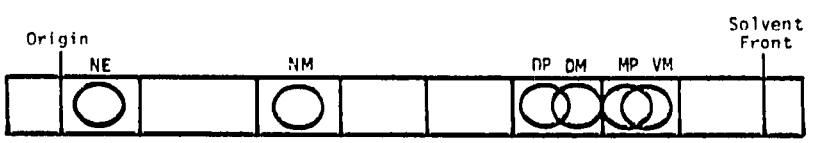
For footnotes, see Table I.

The most striking characteristic of these chromatography papers is that they preferentially retain the amines near the origin. Although no single solvent system adequately separated all six compounds on cellulose phosphate paper (Table II), solvent 14 produced a separation equivalent to that obtained with the column chromatographic method of KOPIN *et al.*[1]. This method separates the compounds into four fractions: NE, NM, the deaminated catechols (DP and DM), and the O-methylated deaminated metabolites (MP and VM).

A complete separation of all six compounds was obtained with a combination of two different solvent systems. Solvent 20 provided a complete separation of the deaminated metabolites of NE with no interference from the amines which were retained at the origin. Solvents 8, 9, 14, 15, and 19 separated NE from NM with no interference from the deaminated metabolites. Solvent 9 appeared to be the best since it provided the greatest separation of NE from NM and an adequate separation of NM from the deaminated metabolites.

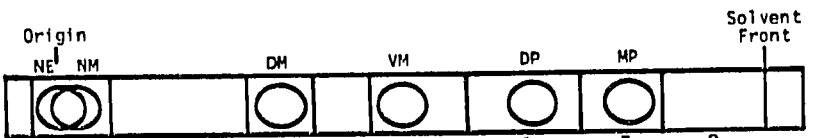
To determine whether there was any significant streaking with these chromatographic systems, ^{3}H-NE, ^{3}H-NM, and ^{3}H-VM were chromatographed in solvents 9 and 20 on cellulose phosphate paper. As illustrated in Fig. 2, there was virtually no cross-contamination of the spots. Essentially all of these compounds were found in their respective spots, except for a small amount of ^{3}H-VM found at the solvent front. When the radioactive peak corresponding to VM was eluted and rechromatographed, the same percentage of radioactivity was still found at the solvent front. This finding suggests that during chromatography some of the ^{3}H-VM decomposes to a substance that runs at the solvent front.

Solvent 9: nBuOH-EtOH-DW = 1:1:1 on Cellulose Phosphate Paper



	n	1	2	3	4	5	6	7	8
Spot ^{3}H-NE	6	96.1 ±0.3	1.1 ±0.3	1.5 ±0.1	0.4 ±0.04	0.3 ±0.0	0.3 ±0.02	0.3 ±0.0	0.1 ±0.0
Spot ^{3}H-NM	8	1.0 ±0.4	1.3 ±0.3	95.5 ±0.7	0.7 ±0.1	0.3 ±0.02	0.3 ±0.03	0.3 ±0.04	0.6 ±0.2
Spot ^{3}H-VM	6	0.1 ±0.01	0.04 ±0.02	0.1 ±0.0	0.1 ±0.02	0.1 ±0.02	5.8 ±0.8	88.4 ±1.1	5.4 ±2.5

Solvent 20: nBuOH-Pyr-DW = 14:3:3 on Cellulose Phosphate Paper



	n	1	2	3	4	5	6	7	8
Spot ^{3}H-NE	6	98.3 ±0.3	0.6 ±0.1	0.2 ±0.02	0.1 ±0.02	0.2 ±0.02	0.3 ±0.02	0.2 ±0.04	0.2 ±0.03
Spot ^{3}H-NM	6	98.0 ±0.3	0.7 ±0.1	0.2 ±0.04	0.1 ±0.0	0.2 ±0.03	0.2 ±0.03	0.3 ±0.05	0.5 ±0.03
Spot ^{3}H-VM	8	0.2 ±0.02	0.1 ±0.02	0.2 ±0.02	0.4 ±0.07	92.1 ±1.0	0.8 ±0.04	0.4 ±0.05	5.7 ±1.0

Fig. 2. Cross-contamination of spots on cellulose phosphate paper with (a) solvent 9 and (b) solvent 20. Values are net c.p.m. in each segment expressed as a percent of total net c.p.m. on chromatogram; mean ± S.E.

In a further attempt to find a single solvent system which would separate all six compounds, several solvent systems were altered as described for Whatman No. 1 paper. In addition, all of the solvent systems tested on cellulose phosphate paper were also tested on carboxymethyl cellulose paper, another cation-exchange paper. No single solvent system tested adequately separated all six compounds on either cation-exchange paper. These data will also be supplied on specific request.

Effect of sodium chloride on the separation of NE and its metabolites

Relatively small amounts of sodium chloride or potassium chloride increase the R_F values of NE and NM on the cation-exchange papers in some solvent systems. In the experiments summarized in Table III, NE and its metabolites were spotted on the chromatography paper. After the spot had dried, 1.7 μmole NaCl (5 μl of 2% NaCl) was added. This small amount of NaCl increased the R_F value of NE and NM on cellulose phosphate and on carboxymethyl cellulose paper in solvent 20. There was no increase in the R_F values of the amines on Whatman No. 1 paper or silica gel loaded paper, nor was there any increase in the R_F values of the deaminated metabolites on any of the chromatography papers. This amount of NaCl did not increase the R_F value of any of the compounds in solvent 9.

Table IV shows the effect of increasing amounts of NaCl on the R_F value of

TABLE III

EFFECT OF 1.7 μMOLES SODIUM CHLORIDE ON THE $R_F \times 100$ VALUES OF NOREPINEPHRINE AND ITS FIVE MAJOR METABOLITES

Solvent No.	*Paper*	*μmole NaCl*	*$R_F \times 100$ value*					
			NE	*NM*	*DP*	*DM*	*MP*	*VM*
20	Whatman No. 1	0	26	31	61	6	71	10
20	Whatman No. 1	1.7	25	30	62	8	72	13
20	Cellulose phosphate	0	0.6	3	66	29	81	49
20	Cellulose phosphate	1.7	2	20[d]	67	31	82	51
9	Whatman No. 1	0	54	64	70	38	80	48
9	Whatman No. 1	1.7	55	65	70	39	78	50
9	Cellulose phosphate	0	5	29	75	68	86	80
9	Cellulose phosphate	1.7	4	29	76	67	87	81

For footnotes, see Table I.

TABLE IV

EFFECT OF VARIOUS AMOUNTS OF SODIUM CHLORIDE ON THE $R_F \times 100$ VALUE OF NORMETANEPHRINE

Solvent No.	*Paper*	*μmole NaCl*					
		0	*0.17*	*0.51*	*1.7*	*5.1*	*17*
20	Whatman No. 1	35	37	36	33	36	34
20	Cellulose phosphate	2	3	4	20[d]	20[d]	21[d]
9	Whatman No. 1	64	64	64	64	65	65
9	Cellulose phosphate	32	30	31	30	30	35[d]

For footnotes, see Table I.

NM. In solvent 20, as little as 0.51 μmole NaCl increased slightly the R_F value of NM on cellulose phosphate paper. Although lower amounts of NaCl did not affect the R_F of NM in solvent 9, 17 μmole NaCl definitely increased the R_F value on cellulose phosphate paper. However, even this amount of NaCl did not increase the R_F value of NM on Whatman No. 1 paper in either solvent.

DISCUSSION

No single solvent system tested adequately separates NE and all five of its major metabolites. All together, 111 solvent systems were tested on Whatman No. 1 paper; 8 solvent systems were tested on acid-washed Whatman No. 1 paper; 56 solvent systems were tested on silica gel loaded paper; and 62 solvent systems were tested on both cellulose phosphate and carboxymethyl cellulose papers. A complete separation of all six compounds was obtained by using a combination of solvent systems 9 and 20 on cellulose phosphate paper. These two solvent systems can be used to develop a single sheet of paper by two-dimensional chromatography. However, for routine analysis of a large number of samples, the author prefers to spot half of the sample on each of two strips of cellulose phosphate paper. One strip is developed in solvent 9 and the other in solvent 20. In this way, a greater number of samples can be chromatographed in both solvents simultaneously and a smaller amount of chromatography paper is required. An assay for ^{3}H-NE and its metabolites based on this chromatographic technique has been developed and is currently being tested to make sure that it will provide reliable data.

The negative data presented in this paper are also noteworthy. PC and thin-layer chromatography are used extensively to test the purity of radioactive catecholamines and their metabolites in various types of samples, *e.g.*, fractions obtained by column chromatography. Many of the solvent systems listed in Table I have been used with Whatman No. 1 paper for this purpose. In many of these solvent systems, two or more of the metabolites have essentially the same R_F value. Great care must be exercised in the selection of a solvent system for this purpose. A solvent system is only useful for checking the purity of a given sample if it is known to separate the compound of interest from all potential impurities. For use with NE and its metabolites, the author strongly recommends the use of solvents 9 and 20 on cellulose phosphate paper. No other chromatographic technique tested provided a comparable separation of all six compounds.

The major difficulty encountered in the use of cellulose phosphate paper is that a small amount of sodium or potassium chloride will increase the R_F value of NM in solvent 20 on cellulose phosphate paper. This effect is probably due to competition for the anionic sites on the paper. If the R_F value of NM is increased too much, there may be some cross-contamination of the DM spot. Since sodium and potassium chloride are found in virtually all biological samples, it is necessary to ensure that the amount present in a given sample does not prevent adequate separation of NM and DM.

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