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A MODIFIED PAPER CHROMATOGRAPHIC METHOD FOR ASSAY OF MYOCARDIAL--ETC(U)
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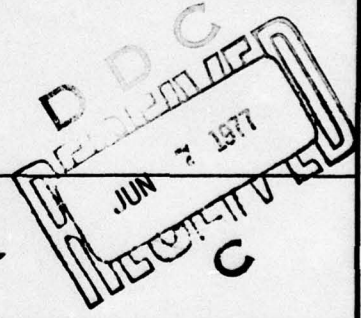
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A570 of the eluted serine standard. A direct linear relationship was shown between MDF chromatographic and bioassay values for each animal. This technique for paper chromatographic assay of MDF is as sensitive, specific, and reliable as bioassay, but offers clear advantages of small sample volume requirement, reduced analysis time, and ease of performance with routinely available laboratory equipment.

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A simplified paper chromatographic technique for assaying sporadic depression factor (SPF) is presented. In addition to a serine standard, dephosphorylated plasma samples from rabbits subjected to neurologic shock were applied to paper and chromatographed. The spot in the chromatogram occurring at a migration distance of 1.0 to 1.8 times that of the standard was found by bioassay to contain the SPF. The chromatographic spots were developed with ninhydrin, eluted with 2 ml of 10 mM NaOH and 570 of each eluate was measured and compared against

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A Modified Paper Chromatographic Method for Assay of
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Running Head: Paper Chromatographic MDF Assay

In conducting the research described in this report, the investigators adhered to the "Guide for the Care and Use of Laboratory Animals," as promulgated by the Committee on Revision of the Guide for Laboratory Animal Facilities and Care of the Institute of Laboratory Animal Resources, National Research Council. The facilities are fully accredited by the American Association for Accreditation of Laboratory Animal Care.

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ABSTRACT

↳ A simplified paper chromatographic technique for assaying myocardial depressant factor (MDF) is presented. In addition to a serine standard, deproteinized plasma samples from rabbits subjected to hemorrhagic shock were applied to paper and chromatographed. The spot in the chromatograms occurring at a migration distance of 1.6 to 1.8 times that of the standard was found by bioassay to contain the MDF. The chromatographic spots were developed with ninhydrin, eluted with 3 ml of 1% NaHCO_3^m and A_{570} $\times A(570 \text{ nm})$ of each eluate was measured and compared against A_{570} of the eluted serine standard. A direct linear relationship was shown between MDF chromatographic and bioassay values for each animal. This technique for paper chromatographic assay of MDF is as sensitive, specific, and reliable as bioassay, but offers clear advantages of small sample volume requirement, reduced analysis time, and ease of performance with routinely available laboratory equipment.



INTRODUCTION

Myocardial depressant factor (MDF) was initially identified by Brand and Lefer in the plasma of cats subjected to hemorrhagic shock (1). Since then, it has been described in many forms of shock in a variety of animals as well as in man (2-8). It has been identified as a small polypeptide, having a molecular weight of 500-1000 daltons (9), which is present in blood at a concentration of 0.5-1.0 n mol/ml during shock (10). Assay of MDF has relied upon its ability to depress electrically stimulated contractions of isolated papillary muscle preparations (11,12). This bioassay technique, though dependable, has severe limitations. Both the assay procedure and the preparation of samples for assay are time-consuming and technically complex. In addition, complicated and expensive equipment are required. The number of samples which can be assayed at one time is limited by the viability of the papillary muscle utilized. Furthermore, the relatively large quantity of plasma that is required for the assay limits both the number of samples which can be analyzed in any serial study and the minimum size of the animal used.

Barenholz et al. (13) have described two chemical methods for the determination of MDF in plasma. These methods offer advantages over the bioassay technique but some problems remain. The first method involving spectrofluorometric analysis of deproteinized plasma is sensitive but lacks specificity and requires access to a spectrofluorometer. The second method using paper chromatographic assay techniques is highly specific but also requires access to an expensive and complicated instrument, an integrating densitometer.

A modification of the paper chromatographic assay technique for

the quantitation of MDF is presented herein. The method is simple to perform and requires minimal laboratory equipment. Furthermore, many samples can be tested at one time and only small quantities of plasma are required for assay.

METHODS

Animal Procedures

Adult New Zealand white rabbits weighing 2.0 to 5.0 kg were anesthetized with pentobarbital sodium (20 mg/kg, intraperitoneally), administered heparin (1000 units/kg, intravenously), and subjected to hemorrhagic shock in the manner described by Lefer and Martin (1). The rabbits were bled incrementally through a femoral arterial cannula into a reservoir, and a constant systemic arterial blood pressure of 40 mm Hg was maintained. Following return of 40% of the maximal shed volume to the animal (4 to 6 hours) the remaining blood in the reservoir was reinfused. When arterial pressure dropped to 60 mm Hg (30 to 90 minutes following reinfusion) 40 ml of blood was removed from the animals and processed as described below. Other rabbits were subjected to a "sham" procedure in which femoral artery cannulation was performed and pentobarbital anesthesia was maintained for 4 hours but no blood was shed until the termination of the experiment when 40 ml were removed and processed.

Processing of Plasma

Blood was drawn into plastic syringes containing 10 units/ml of heparin and centrifuged at 2000 g for 10 minutes at 4C. The plasma supernatant was divided into four aliquots: (a) 5 ml were ultrafiltered through a Pellicon filter (Millipore Corporation, Bedford, Mass.) with an exclusion limit of 20,000 daltons, (b) 2 ml were subjected to pressure dialysis through cellulose dialysis tubing (3/8" flat width) under a pressure of 15 cm H₂O until roughly 0.5 ml of dialysate was collected (15 to 20 min), (c) 1 ml was deproteinized using the method of Barenholz et al. (13) by precipitation of plasma proteins with 50% trichloroacetic

acid (TCA) followed by repeated ether extractions, and (d) 10 ml were deproteinized by the TCA-ether method, lyophilized to dryness, reconstituted to 2 ml with distilled water, filtered through a Biogel P-2 column as described by Lefer and Martin (12), and the A_{230} peak occurring at elution volume 85 to 105 ml, which was found to contain all the MDF activity, was collected. All processed samples were stored at -70°C until time of assay.

Bioassay

The samples obtained by gel filtration were bioassayed by the method of Lefer and Martin (12) on papillary muscles removed from the right ventricles of anesthetized rabbits. Muscles were stimulated to contract at 1 beat/second using a voltage 20% above threshold; maximal developed muscle tension was obtained by appropriate adjustments to resting tension. Each sample was bioassayed on at least two papillary muscles. One MDF unit equals 1% depression in developed tension as compared to tension developed in Krebs-Henseleit solution. In calculating MDF bioassay units, a correction was made for the dilution of plasma during gel filtration. For example, if an initial 10-ml plasma sample was processed and applied to the column and the final volume of the eluted fraction containing the MDF peak was 20 ml, a 2-fold dilution, the MDF bioassay value measured for the filtrate was multiplied by a factor of 2. Since a direct linear relationship between concentration of sample and percent inhibition of papillary muscle contraction has been established previously (11), this correction was considered valid. Hence, all MDF bioassay results are reported as units/10 ml of initial plasma sample.

Paper Chromatographic Assay

Samples of plasma (200 μ l) processed by all four techniques described above were applied to Whatman's 3 MM paper (57 x 23 cm) in repeated 10- μ l aliquots and dried with a warm air blower so that the total area of application for each sample did not exceed 0.5 cm². Twenty microliters of a 1-mg/ml solution of L-serine was applied to each paper as a standard. As described by Barenholz et al. (13), the papers were equilibrated in a tank saturated with a solvent consisting of n-butanol, glacial acetic acid, and double distilled water in a 25:25:6 v/v/v ratio. Following application of solvent for 18 hours via descending flow, each chromatogram was suspended in room air until dry, sprayed with a ninhydrin aerosol containing 0.2% ninhydrin and 2.5% 2,4,6-trimethylpyridine in 2-propanol (J. T. Baker, Phillipsburg, N.J.) and incubated at 90°C for 15 minutes. The spot, containing essentially all the MDF activity when eluted, was found at a migration distance roughly 1.6 to 1.8 times the distance that serine had migrated ($R_s = 1.6$ to 1.8). This spot [corresponding to spot G as identified by Barenholz et al., (13)], the serine spot, and, as a blank, a comparable sized piece of the chromatogram on which no color could be visualized were cut from the papers. The cut spots were fan-folded and placed in the bottom of 15 ml test tubes. The purple color of the spots was eluted by adding 3 ml of 1% NaHCO₃ to each test tube and incubating in a water bath at 60°C for 15 minutes. The folded papers were discarded, the remaining solution was centrifuged at room temperature for 10 minutes at 2000 g, and absorption of supernatant was read at 570 nm in a Beckman DB-G spectrophotometer. One MDF chromatographic unit was equal to A₅₇₀ of 1.3 nmoles of serine.

Special Procedure

In order to measure bioassayable MDF activity of eluted paper chromatographic spots, relatively large quantities of MDF were required. Thus, incubated homogenates of rabbit pancreas prepared by the method of Herlihy and Lefer (14) were used rather than plasma. An homogenate of pancreata from 4 rabbits was deproteinized and applied to a Biogel P-2 column as described above. The entire fraction containing the MDF peak (elution volume 85 to 105 ml) was applied to sheets of Whatman's 3 MM paper in 150- μ l aliquots spaced 1 cm apart. The papers were chromatographed but they were not developed with ninhydrin. A reference chromatogram containing a serine standard spot and multiple sample spots was made simultaneously and developed with ninhydrin. Strips of paper corresponding to each of the major spots on the reference chromatogram were cut from the undeveloped chromatograms. The strips were cut into pieces 3 cm in width, folded, and incubated in 5 ml of 1% NaHCO₃ at 60°C for 15 minutes. The eluates from each strip were pooled, lyophilized, and filtered again through a Biogel P-2 column. The fraction obtained at elution volume 85 to 105 ml containing the single A₂₃₀ peak was bioassayed.

RESULTS

In order to determine the location of MDF activity on paper chromatograms, the major visible spots were eluted from the papers and bioassayed. The results are recorded on Table 1. Four major spots were noted; I, II, III, and IV with respective R_s values of 0.5 to 0.7, 1.0 to 1.4, 1.6 to 1.8, and 2.0 to 2.4. Spots I, II, and IV exhibited a minimal amount of MDF activity but spot III contained the major fraction. Furthermore, there was no consistent relationship between the bioassayable activities of spots I, II, and IV and their paper chromatographic values, but there was for spot III. Thus, all paper chromatographic measurements for MDF in this study were performed on spot III alone.

Various amounts of processed plasma were applied to paper chromatograms in order to assess the relationship between sample volume and measured MDF. As shown in Figure 1, there was a direct linear relationship between the two parameters which could be expressed by the equation $y = 1.06x - 4.8$ with y representing MDF chromatographic units and x representing sample volume. A coefficient of correlation equal to 0.914 was obtained. Mean MDF bioassay activity was 22 ± 2 units/10 ml (\pm SE) for control rabbits and 54 ± 7 units/10 ml for shock rabbits. These results were similar to those obtained by others in cats.

Four different methods of processing plasma for paper chromatography were tried in order to determine the relative reliability, ease, and time-consumption of each. Comparison of the four methods is depicted on Table 2. As can be seen, there was no significant difference between the actual values obtained by paper chromatography from plasma processed by any of the methods, however, a predictable general trend was observed. Biogel P-2 filtration and Pellicon ultrafiltration resulted in somewhat reduced

values when compared to TCA precipitation-ether extraction. This was not surprising since both filtration gels and membranes for ultrafiltration tend to bind polypeptides and proteins. Pressure dialysis, on the other hand, produced generally higher values than TCA-ether treatment, probably reflecting the inevitable loss of small amounts of sample during the latter procedure. Pressure dialysis also proved to be the most rapid method of processing plasma. Enough sample for chromatography could be obtained by pressure dialysis within 20 minutes as compared to up to 4 hours required for TCA-ether treatment.

DISCUSSION

The modified paper chromatographic method for measuring MDF herein presented is simple and highly specific. This method is relatively rapid and requires a minimum of laboratory equipment. It permits many samples to be assayed at one time and only small amounts of sample are required; thus, this technique is particularly useful in serial studies involving small animals.

There was no significant difference between measured MDF values using TCA precipitation and ether extraction, Biogel P-2 filtration, Pellicon ultrafiltration, or pressure dialysis in processing plasma for paper chromatographic assay. However, pressure dialysis was the quickest and simplest to perform and required the least amount of sample, thus this deproteinization technique was preferred over the others.

In order to properly evaluate the paper chromatographic MDF assay technique, a rabbit shock model was employed and a bioassay technique utilizing rabbit papillary muscles was established for comparison (15). Rabbits subjected to hemorrhagic shock in the manner described by Lefer and Martin (12) responded quite similarly to cats, and the time course for the shock procedure was similar. Although no previous data regarding MDF in shock rabbits are available, the results obtained for both control and shock rabbits did not differ greatly from those previously reported in cats (12), dogs (16), and humans (8).

A wide range of MDF was recorded for the control and shock animals, thus facilitating comparison of a bioassay with paper chromatography. The direct linear relationship that was established between the two assay techniques and the excellent coefficient of correlation indicate that paper chromatographic MDF measurements accurately represent bioassayable MDF activity. Results obtained by eluting the major spots

from the chromatograms and measuring their activities by bioassay suggest that spot III contained essentially all of the MDF initially applied to the papers. This observation is in agreement with Barenholz *et al.* (13) who found that their spot G (also at Rs 1.6 to 1.8) contained the MDF.

The sensitivity of the paper chromatographic assay is reflected not only in its close correlation with bioassay but also in the linear relationship between the amount of sample applied to paper and measured MDF. It would be safe to conclude that paper chromatography is at least as sensitive as bioassay in measuring plasma MDF. The small standard deviation observed in multiple measurements of a single sample, particularly when optimum 200- μ l aliquots were applied (SD < 5%) points to the excellent reproducibility of the assay, again comparable to that of bioassay.

The greatest advantage that assay of MDF by paper chromatography offers is in its convenience and ease. As little as 0.5 ml of initial plasma sample is enough to perform a duplicate reading by paper compared to a minimum of 10 ml plasma required for each bioassay (12). This opens up a number of possibilities for evaluation of the role of MDF in various pathologic states. The paper chromatographic technique has been utilized in our laboratory to assess the role of MDF in shock produced by staphylococcal enterotoxin B in small rhesus monkeys (17). Over a 24-hour period it was possible to withdraw and measure MDF in 12 samples from a single monkey without seriously affecting its blood volume.

The bioassay technique for measuring MDF involves gel filtration of each plasma sample for up to 24 hours. Since each column requires a fraction collector and it is not generally feasible to operate more

than two columns at one time, processing of more than a few samples can take weeks. On the other hand, using pressure dialysis, as many as 20 samples were processed in our laboratory within 15 minutes. Solvent saturation of papers for chromatography was somewhat time-consuming (18 hours). However, no specific care was required during the procedure; samples were usually applied in the afternoon and the papers were ready for analysis the following morning after saturating overnight. As many as eight samples were applied to a single sheet of paper and up to six papers were chromatographed each day. In contrast, no more than 12 processed samples could be bioassayed in a day and the procedure required the continuous presence of an experienced observer.

Barenholz et al. (13) introduced two chemical methods of assaying MDF. The authors described a relatively sensitive and rapid spectrofluorometric assay; however, its usefulness was severely limited by its lack of specificity as well as its requirement for an expensive piece of equipment, a spectrofluorometer. Their paper chromatographic method was highly sensitive and specific but required an integrated densitometer, an instrument not commonly available. The modified paper chromatographic assay technique presently described circumvents the requirement for a densitometer and can be performed with routinely available equipment. Furthermore, it simplifies the procedure so that a larger number of samples can be processed in a shorter time while decreasing the minimum sample volume required.

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TABLE I. Bioassayable MDF Activity of Major Spots Eluted from Paper Chromatograms

Spot designation	Rs	MDF units
I	0.5 - 0.7	16
II	1.0 - 1.4	22
III	1.6 - 1.8	67
IV	2.0 - 2.4	25

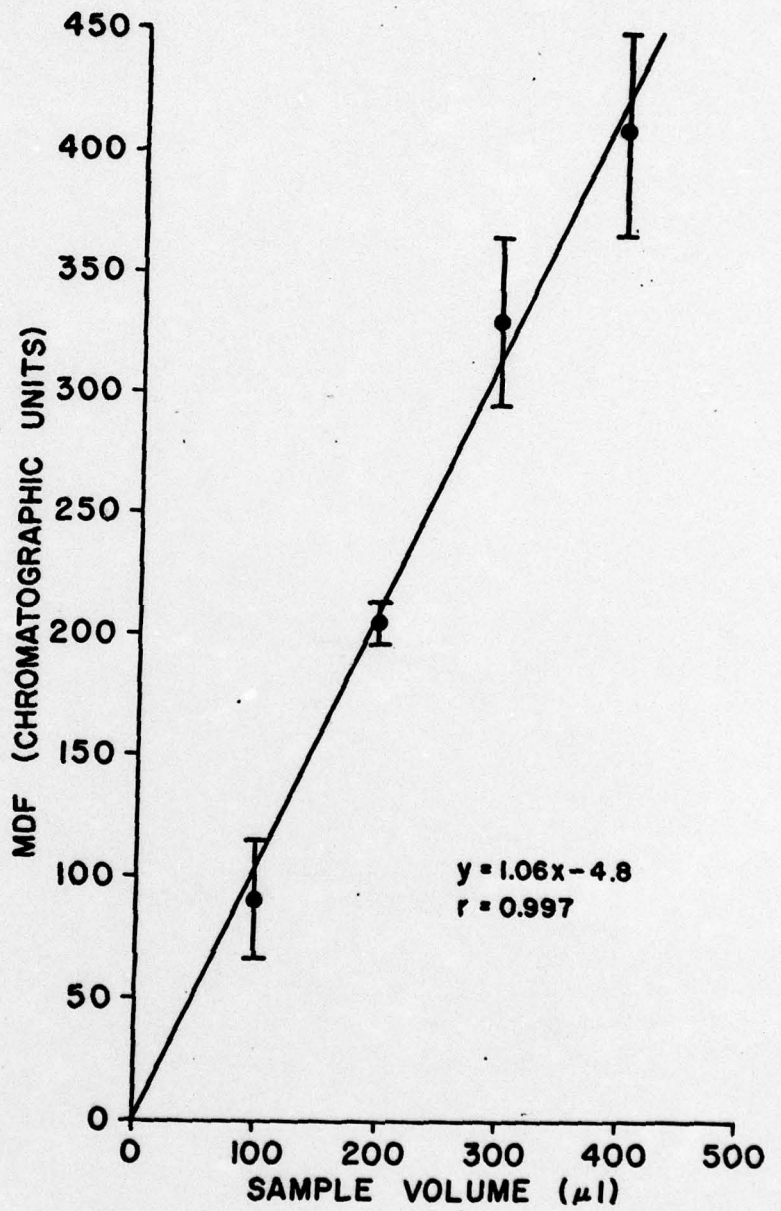
TABLE II. Comparison of Various Methods of Processing Plasma for Paper Chromatography

Sample Number	Y MDF (units/10 ml) Bioassay	X MDF (chromatographic units/ μ l)			
		TCA precipitation ether extraction	Biogel P-2 gel filtration	Pellicon ultrafiltration	Pressure dialysis
1	84	1.95	1.60	1.72	2.10
2	30	1.00	0.86	0.80	1.24
3	17	0.78	0.70	0.62	1.00
4	42	1.26	0.98	0.90	1.40
5	50	1.83	1.60	1.49	2.04
6	32	1.06	0.92	0.79	1.16
7	80	3.08	2.63	2.30	3.29
Slope of X vs. Y (Slope \pm SE)		28.3 \pm 6.8	32.3 \pm 8.7	38.1 \pm 6.9	27.5 \pm 7.1
Length of procedure (hr)		3.0 - 4.0	20.0 - 24.0	0.2 - 0.5	0.2 - 0.3

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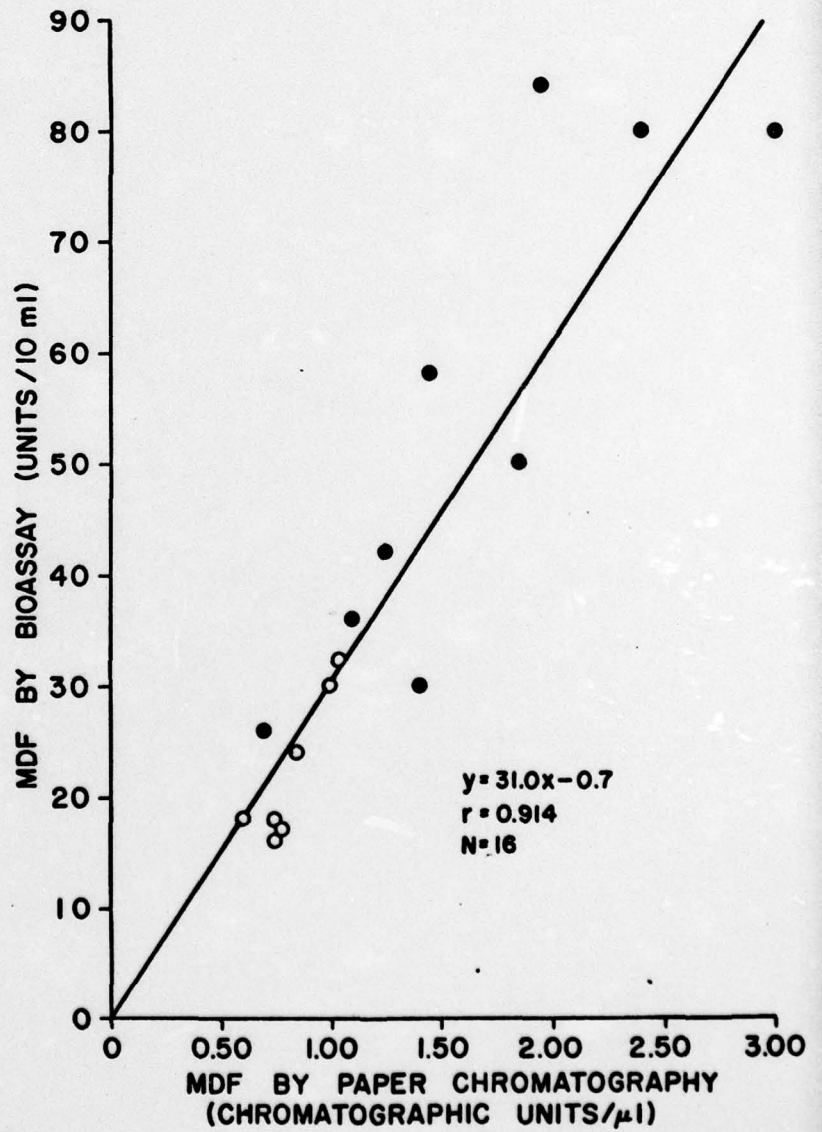
Figure 1. Graph of total MDF value obtained by paper chromatography vs. volume of sample initially applied to paper. Each point represents mean \pm standard deviation of 6 values.

Figure 2. Comparison of bioassayable MDF activity against MDF obtained by paper chromatography. The closes circles (●) represent shock rabbits and the open circles (○) controls.



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FIGURE 1. Graph of total MDF value obtained by paper chromatography vs. volume of sample applied to paper.

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FIGURE 2. Comparison of bioassay-
able MDF activity vs. MDF obtained
by paper chromatography.

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