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Note

Improved thin-layer chromatographic system for methadone and its metabolites in biological samples

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In our studies of the metabolism and physiological disposition of methadone we found that the Gelman instant thin-layer chromatography (ITLC) system reported by Misra *et al.*[1] offered the most functional separation of methadone and its metabolites. The silica gel-impregnated glass fiber plates were easy to cut apart and place in separate counting vials to be assayed for radiolabeled drugs and metabolites. Our main problem with this ITLC system was the tendency to overload when working with biological extracts. To overcome this problem, a thicker layer of silica gel was placed at the base of the impregnated strips. We are aware of a similar application of gel to thin-layer plates, but we believe ours is the first application of gel to silica gel-impregnated fiber glass strips.

We have applied the procedure successfully to many tissues including brain, blood, fat, feces, kidney, lung, muscle, and spleen with greatly improved selectivity and sensitivity.

MATERIALS AND METHODS

l-Methadone-1-^{3}H · HB_r (135mCi/mmole) was purchased from New England Nuclear (Boston, Mass., U.S.A.). Samples of *d,l*-2-ethyl-1,5-dimethyl-3,3-diphenyl-1-pyrroline · HCl and *d,l*-2-ethylidene-1,5-dimethyl-3,3-diphenyl-1-pyrrolidinium perchlorate were kindly furnished by Dr. I. F. Bennett of Eli Lilly & Co. (Indianapolis, Ind., U.S.A.); methadone N-oxide was a kind gift from Dr. A. H. Beckett of Chelsea College (London, Great Britain). Standard ITLC Type S.G. 5 cm × 20 cm chromatography media were obtained from Gelman (Ann Arbor, Mich., U.S.A.).

Each chromatographic strip was dipped in a silica gel slurry consisting of silica gel (Brinkmann, Westbury, N.Y., U.S.A.), anhydrous $CaSO_4$ and water (20 g, 6 g, and 60 ml, respectively to a height of approximately 2 cm. The strips were then placed in an oven (100–105°) and held until use. The dried silica gel was removed from the back and from the lower 0.5 cm of the front of the strip. The upper and lower borders of the applied layer were tapered. The procedure resulted in a final layer thickness of 1–2 mm.

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Analysis of a biological sample was done by extracting a 4-l tissue homogenate with methanol, then extracting the dried methanol extract with diethyl ether and carbonate buffer at pH 10. The extract was then spotted in the center of the applied layer, with known standards and developed in three chromatographic systems.

The first system consisted of ethyl acetate–methanol–concentrated NH_4OH (85:10:5) and was developed five times to a height of 4 cm from the origin. The second system consisted of benzene–ethyl acetate (95:5) and was run once to a height of 17 cm from the origin. The third system consisted of benzene–ethyl acetate–methanol–concentrated NH_4OH (80:20:1.2:0.1)[1] and was developed 17 cm from the origin. After each development the strips were blown to dryness under gentle heat. Visualization resulted with iodoplatinate spray as described by Stahl[2].

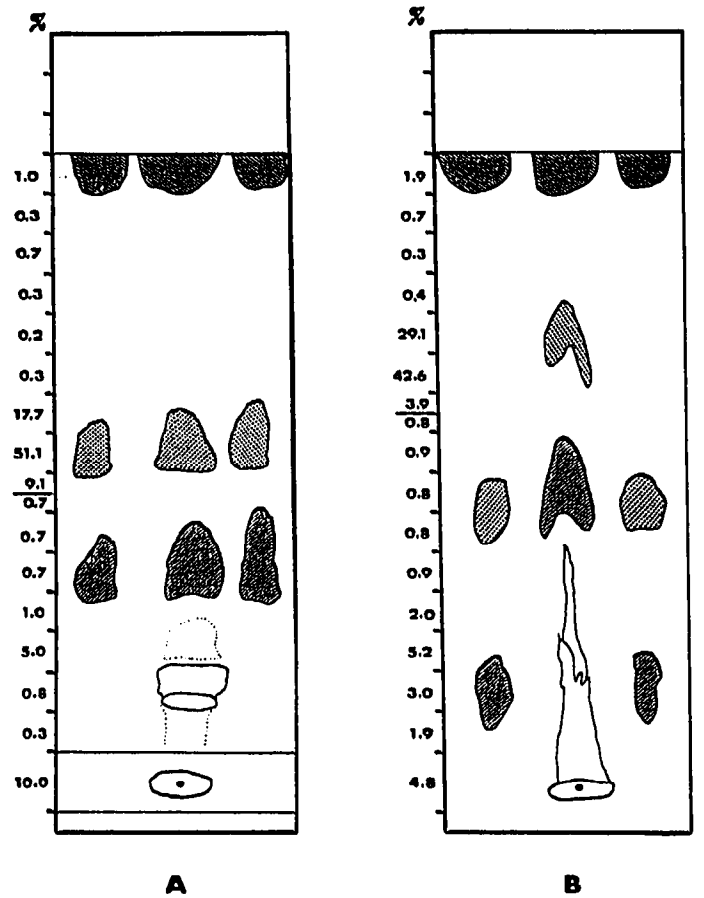

Fig. 1. Separation of a kidney extract. (A) Silica gel applied to Gelman ITLC plate. R_F = 0.00 unknown polar metabolite; R_F = 0.23, methadone N-oxide; R_F = 0.36, pyrrolidine metabolite; R_F = 0.55, methadone; R_F = 0.97, pyrroline. 36,961 dpm/plate. (B) Standard ITLC plate. R_F = 0.00, unknown polar metabolite; R_F = 0.16, pyrrolidine standard; R_F = 0.44, methadone standard; R_F = 0.47, pyrrolidine from biological extract; R_F = 0.72, methadone from biological extract; R_F = 0.97, pyrroline. 31,348 dpm/plate.

RESULTS AND DISCUSSION

The first chromatographic system separates methadone, pyrroline and pyrrolidine metabolites, and methadone-N-oxide from the origin. The repeated development is necessary to insure complete separation of these compounds from the origin, due to the increased absorptivity and subsequent retentiveness of the silica gel layer as compared to the unaltered ITLC strip. Furthermore, this step leaves methadone and these metabolites in a narrow band, 4 cm from the origin, optimizing their separation by the final chromatographic system. The second system is most functional in tissues having a large amount of lipid constituents, moving lipids to the solvent front. The final chromatographic system, which resolves methadone and its metabolites, is the separation system first reported by Misra *et al.*[1].

We found it necessary to develop the strips to a height of 17 cm from the origin to insure an adequate separation between methadone (R_F 0.55) and pyrrolidine (R_F 0.36). Pyrroline runs with the solvent front and methadone-N-oxide runs at R_F 0.23. These R_F values result from development in the three development systems.

The results of a kidney extract assay, including the percentage of radioactivity and total dpm are shown in Fig. 1. Approximately 5 mg of a "dried" resinous extract were spotted in benzene–methanol (1:1) on each strip, one with the added gel layer, one without the gel layer. The chromatographic strip without the gel layer was developed with the third system only.

This improved system resulted in good separation with close approximation between the biological sample and the standards; in addition, separation was possible between two labeled areas, one on the origin and one at R_F 0.23, which corresponds to methadone-N-oxide. This resolution had not been possible with the overloaded system. The persistence of radioactivity held at the origin after numerous chromatographic developments is interesting. Misra and Mulé[3] reported a "persistent association of radioactivity with brain debris", for which they suggested a covalent linkage with protein. We have noticed this persistent activity at the origin[4] in numerous tissue types.

We believe that the increased resolution and greater sensitivity make this system superior to existing ones when it is applied to assays of methadone and its metabolites, and that it should be studied for future adaptation to other thin-layer chromatographic analyses.

REFERENCES

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4 C. M. Davis and D. C. Fenimore, unpublished results.