Rapid Identification of Analgesic Drugs in Urine with Thin-Layer Chromatography

The separation and identification of analgesic drugs, both synthetic and those derived from opium, through the use of conventional paper chromatography has already been described[1,2]. However, for some purposes, the length of time required for the development of paper chromatograms (12–20 h) is a distinct disadvantage. Newer methods of chromatographic analysis, such as thin-layer chromatography (Dünnschicht-Chromatographie)[3–7], reduce the time required at least 10-fold ($^1/_2$–2 h) and should therefore be very useful in toxicology and forensic medicine for the separation and identification of narcotic drugs as well as barbiturates, tranquilizers, and related compounds. We shall describe a method which makes it possible to identify and distinguish a number of analgesic drugs in about 4 to 5 h after the time of collection of the urine sample.

Techniques. The extraction of drugs from urine is carried out in the following manner: A sample of urine containing at least 100 µg of drug is adjusted to pH 9.0 with sodium hydroxide and then shaken for 15 min with 4 vol of ethylene chloride containing 10% isoamyl alcohol. After centrifugation, the organic layer is evaporated *in vacuo* and the residue taken up in 1–2 ml of ethyl alcohol, and an aliquot is then chromatographed.

In those instances where acid hydrolysis is necessary to split the free base from the glucuronide, the form in which the majority of the morphine derivatives and synthetic analgesics are excreted in man, a portion of the urine sample is heated for 1 h at 100°C with 1/10 vol of concentrated HCl before being made alkaline and extracted. Most of the alkaloids, including morphine, levorphanol, codeine, and propoxyphene, were stable under these conditions of acid hydrolysis. Heroin was, however, converted to morphine, and phenazocine yielded a small amount of a breakdown product.

Chromatoplates are made as described by STAHL[8] and BRENNER[4], using either silica gel G (25 gm/50 ml) or aluminium oxide G (30 gm/30 ml), Merck, Darmstadt. After the development of the plates as described in the Table, they are briefly air-dried and then dried at 80–100°C for 45 min to remove excess solvent and thereby minimize background colour development. The alkaloids are detected by spraying with potassium iodoplatinate reagent[9].

Results. In the Table are presented the Rf values for a number of drugs which were spotted as the pure hydrochloride salts. The six solvent systems listed in the Table were chosen as those best suited for the separation of analgesic mixtures, and by appropriate selection of these systems positive identification of the components of such mixtures in urine extracts was possible. Thus chromatograms obtained from urines to which were added (1) normorphine, morphine, codeine, chlorpromazine, and heroin, or (2) morphine, meperidine, 6-mono-acetyl morphine, methadone, and tripelennamine, made possible separation and identification of all components in these mixtures. Furthermore, for drugs such as phenazocine and propoxyphene whose Rf's, in one dimension, are almost identical, two dimensional chromatography, using systems S4 and then S1 or S4–S2, clearly separate them. (For the components of the solvent systems see Footnote [a] to the Table.)

One of the chromatograms of a urine extract from a patient receiving morphine, pyridoxine, chloramphenicol, trimethobenzamide, griseofulvin, secobarbital and prednisone is shown in Figure 1. Four iodoplatinate-positive spots were detected and with the other solvent systems described in the Table, two of the spots were identified with certainty as morphine and trimethobenzamide. The other spots were presumed to be metabolites since their Rf's did not correspond to those of the original drugs used as references.

Rf values of various compounds extractable from urine at pH 9.0 that react with iodoplatinate reagent[a,b]

Compound	Rf values silica gel				Rf values alumina	
	S1	S2	S3	S4	A1	A2
1. Mescaline	0.18	0.81	0.30	0.30	0.36	0.53
2. Morphine	0.40	0.55	0.17	0.34	0.59	0.18
3. Normorphine	0.14	0.76	0.05	0.16	0.33	0.11
4. Meperidine	0.51	0.64	0.90	0.44	0.77	0.98
5. Cocaine	0.74	0.45	0.98	0.39	0.61	0.96
6. Heroin	0.47	0.54	0.80	0.43	0.72	0.83
7. Nicotine	0.58	0.27	0.90	0.44	0.72	0.90
8. Codeine	0.40	0.52	0.46	0.32	0.72	0.65
9. Levorphanol	0.34	0.75	0.62	0.29	0.76	0.91
10. Methadone	0.53	0.78	0.96	0.20	0.78	0.98
11. Chlorpromazine	0.48	0.79	0.93	0.35	0.80	0.98
12. Tripelennamine	0.50	0.46	0.93	0.28	0.76	0.98
13. Phenazocine	0.90	0.93	0.93	0.59	0.86	0.98
14. Nalorphine	0.82	0.71	0.34	0.75	0.72	0.20
15. Propoxyphene	0.80	0.82	0.97	0.53	0.84	1.00
16. Dihydro-morphinone	0.19	0.28	0.22	0.18	0.65	0.25

[a] Solvent systems:
S1 = Ethyl alcohol: pyridine: dioxane: water 50:20:25:5
S2 = Ethyl alcohol: acetic acid: water 60:30:10
S3 = Ethyl alcohol: dioxane: benzene: ammonium hydroxide 5:40:50:5
S4 = Methyl alcohol: *n*-butanol: benzene: water 60:15:10:15
A1 = *n*-Butanol: *n*-butyl ether: acetic acid 40:50:10
A2 = *n*-Butanol: *n*-butyl ether: ammonium hydroxide 25:70:5

[b] 5–10 λ of solution containing 5–10 µg of drug are spotted. Solvent front is allowed to rise 10 cm.

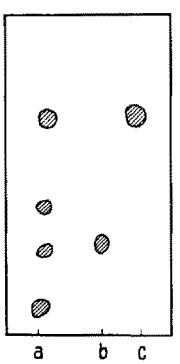

Fig. 1. Chromatogram (silica gel G) solvent S3 of (a) urine extract from patient receiving morphine, trimethobenzamide and other drugs, (b) morphine and (c) trimethobenzamide.

[1] G. J. MANNERING, A. C. DIXON, N. U. CARROL, and O. B. COPE, J. lab. clin. Med. *44*, 292 (1954).
[2] G. R. NAKAMURA, Bulletin on Narcotics *12*, 17 (1960).
[3] E. NÜRNBERG, Arch. Pharm. *292*, 610 (1959).
[4] M. BRENNER and A. NIEDERWIESER, Exper. *16*, 378 (1960).
[5] K. RANDERATH, Angew. Chemie *73*, 436 (1961).
[6] E. STAHL and U. KALTENBACH, J. Chromat. *5*, 458 (1961).
[7] E. DEMOLE, J. Chromat. *1*, 23 (1958).
[8] E. STAHL, Pharmazie *11*, 633 (1956).
[9] To 10 ml of 10% solution of platinum chloride are added 250 ml of 4% potassium iodide and diluted with water to 500 ml.

In addition to morphine and trimethobenzamide, codeine, propoxyphene, meperidine, phenazocine, and levorphanol were identified in the urines of patients receiving one or more of these drugs. A two-dimensional chromatogram is shown in Figure 2 in which development was carried out first with solvent S4 and then with solvent S3, which allowed the separation and identification of each of the components of a ten-component mixture.

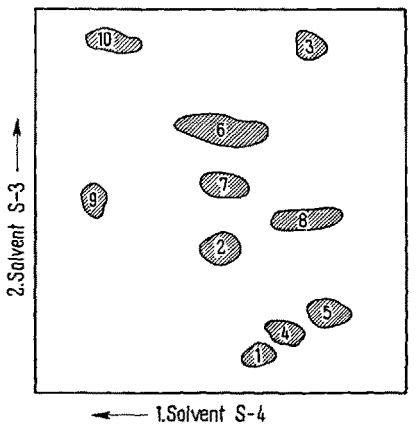

Fig. 2. Two-dimensional chromatogram (silica gel G) of (1) morphine, (2) meperidine, (3) cocaine, (4) codeine, (5) levorphanol, (6)l methadone, (7) chlorpromazine, (8) tripelennamine, (9) nalorphine, and (10) propoxyphene.

Discussion. Thin-layer chromatography was found to be a useful technique for the identification of a variety of drugs in urine extracts. The method is very fast and can be completed conveniently in 5–6 h. It is quite sensitive, detecting 5–10 μg of substance. No interfering substances have been found in control urines which were known to contain caffeine and nicotine[10]. Although the Rf values may be altered by salts and other contaminants of the urine extracts, this can be circumvented by co-chromatographing the reference substances with extracts of control urines. The Rf values were also found to vary somewhat from plate to plate, so that for positive identification it is necessary to chromatograph the reference compounds on the same plate with the unknown mixture.

The patient whose urine was analyzed for morphine had received 15 mg of morphine sulfate parenterally, and the urine was then collected for the subsequent 4 h. Despite the relatively small amount of drug involved and the presence of many other drugs that had been given at the same time, no difficulty was encountered in identifying the morphine excreted, even without hydrolysis of the urine. In the case of addicts taking heroin, identification would be somewhat more difficult because of the small amounts of drug involved, but concentration of large amounts of urine can be effected by flash evaporation.

Thin-layer chromatography is also very useful for *in vitro* and *in vivo* studies of drug metabolism. Such closely related compounds as morphine and normorphine, levorphanol and methorphan, and morphine and dihydromorphinone are easily separated. McMahon[11] has separated propoxyphene and the propoxyphene N-oxide, which were not separable on paper. This technique, with slight modifications in the usual extraction procedure[12], is also useful for the identification of the alkaloids in the opium poppy itself. The analysis of barbiturates and phenothiazene derivatives using the technique of thin-layer chromatography is now under investigation in this laboratory[13].

Zusammenfassung. Es wird eine einfache, schnelle und empfindliche Methode zur Isolierung und Identifizierung von Analgetica mittels der Dünnschichtchromatographie beschrieben. Die Methode ist zur Charakterisierung dieser Stoffe im Harn und für toxikologische, forensische und Stoffwechseluntersuchungen brauchbar.

J. Cochin and J. W. Daly

Laboratory of Chemistry, NIAMD, National Institute of Health, Bethesda (Maryland USA), August 18, 1961.

[10] Nicotine has been identified in very concentrated urine extracts of patients but does not interfere at normal levels.

[11] R. E. McMahon, personal communication.

[12] K. Genest and C. G. Farmilo, Bulletin on Narcotics *8*, 15 (1960).

[13] *Note added in proof:* Direct extraction of tissues (homogenized 1 in 4 with isotonic KCl) and of whole blood has been carried out at pH 9.0 as described for urine. Prior precipitation of proteins is not necessary. Using this procedure, isolation and identification of analgesics added to tissues and blood and of parenterally administered drugs has been effected.

Amethyst Violet as a Stain for Distinguishing Cells with a Damaged Membrane from Normal Cells

In routine serial passage in mice of Ehrlich's ascites tumour cells, it is indispensable to recognize with fairly close approximation the number of live cells inoculated. This need has given rise to various staining techniques for differentiating between live and dead cells. Eosyn Y[1,2] has been used especially and, more recently, nigrosine[3]. The latter, which stains dead or non-vital cells black because of the greater ease in passing through their damaged membranes, is the most widely used at present, also because of its low toxic action.

For the last two years I have successfully used an amethyst violet solution in the routine technique for serial transplantation of Ehrlichs ascites tumour cells. I observed that this stain gives an ever clearer and more constant differentiation between the two kinds of cells; moreover, it is possible to obtain a supra-vital staining of leukocytes. Independently of their vitality, they are more permeable to the stain and it is therefore easier to exclude them, together with the dead cancer cells, from cellular counts of ascites fluid.

Amethyst violet is a basic stain (contrary to eosin Y and nigrosine, which are acid stains), belonging to the azynes series. Its molecular weight is 434.997. It was used by Brenner[4] for supravital staining of mitochondria.

[1] H. M. Patt, M. E. Blackford, and R. L. Straube, Proc. Soc. exp. Biol. Med. *80*, 92 (1953).

[2] R. Schreck, Amer. J. Cancer *28*, 389 (1936).

[3] J. P. Kaltenbach, Merle H. Kaltenbach, and W. B. Lyons, Exp. Cell Res. *15*, 112 (1958).

[4] S. Brenner, Stain techn. *163* (1950).