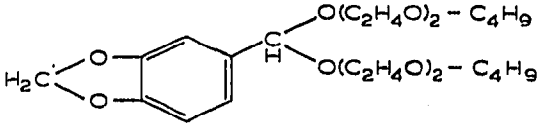
THIN-LAYER CHROMATOGRAPHY OF RAT BILE AND URINE FOLLOWING INTRAVENOUS ADMINISTRATION OF TROPITAL-METHYLENE-^{14}C

L. FISHBEIN* AND J. FAWKES
Bionetics Research Laboratories, Falls Church, Va. (U.S.A.)
AND
H. L. FALK AND S. THOMPSON
National Cancer Institute, Bethesda, Md. (U.S.A.)
(Received May 25th, 1967)

In an earlier paper[1] we reported on the elimination of pesticidal synergists (piperonyl butoxide and tropital)[2] and their metabolites in rat bile and urine, resulting from single intravenous administration of the above compounds.

The purpose of this investigations was to further elaborate the nature of the metabolites of tropital-methylene-^{14}C (I)** in rat bile and urine by thin-layer chromatographic analysis.



(I)

EXPERIMENTAL

Solvent systems

(A) Toluene–acetic acid–water (10:10:1)
(B) Ethyl acetate–acetic acid–methanol (70:10:20)
(C) Hexane–ether (1:3).

Detecting reagents

(1) Chromogenic agent: chromotropic acid reagent[3].
(2) Radiation source: U.V. 2537 Å Mineralight, Model UVS-11***.

Bile and urine sampling

Single intravenous injections of 0.05 ml ($2.575 \cdot 10^8$ c.p.m.) of tropital-methylene-^{14}C were given to adult male rats of the Sprague-Dawley strain averaging 290 g in weight. Bile samples were collected by fistula and urine samples by cannulation from each rat. Details on the handling of the animals, anesthesia, surgery, sample collection and timing have been described previously[4].

* Present address: National Environmental Health Sciences Center, Research Triangle Park, N.C., U.S.A.
** Obtained from McLaughlin, Gormley King Co., Minneapolis, Minn., U.S.A., in 94% radiochemical purity (4% piperonal and 2% unknown).
*** Obtained from Allied Impex Corp., New York, N.Y., U.S.A.

At least three urine samples were collected, one before i.v. injection, a second at an appropriate interval after injection and a third sample at the termination of the bile collection. All samples were kept frozen until ready for analysis.

Chromatography

Twenty microliters of all bile and urine samples were applied as half-inch streaks* on Silica Gel DF-5 plates, prepared as described previously[1]. Bile samples were developed with toluene–acetic acid–water (10:10:1) and urine samples with the ethyl acetate–acetic acid–methanol (70:10:20) solvent system. Each developed plate was photographed in color under 2537 Å illumination. Four-day autoradiograms were then made of each plate. Finally each plate was sprayed with chromotropic acid reagent, then heated at 120° for 5–10 min to develop the color and photographed using equipment and procedures described previously[1].

Preparation of autoradiograms

Four-day autoradiograms were prepared by sandwiching 6.5 × 8.5 Kodak Medical X-ray (No screen) Safety film** between the chromatoplates and a glass plate, then taping the two plates together. Films were developed with Kodax X-ray developer and fixed with regular Kodax fixer using the recommended specifications of the manufacturers.

RESULTS AND DISCUSSIONS

Selected bile samples from a rat injected i.v. with tropital-methylene-^{14}C were developed with toluene–acetic acid–water (10:10:1). The metabolites, visible under 2537 Å and detected with chromotropic acid spray in an ancillary development, were compared with radioactive metabolites as indicated by autoradiograms in this study. Figs. 1a and 1b (depicting two time periods, *e.g.* 10–86 min and 181–265 min) are *illustrative* of the series of contact prints of the 4-day autoradiograms covering the total bile collection period of 10.3 to 560 min after i.v. administration of tropital-^{14}C. Metabolite numbers listed on the left side in these figures indicate each radioactive compound.

Table I gives a composite summary of rat bile R_F values absent in control bile, estimated isotope activity, their color development and intensity, and the time of metabolite appearance and persistence, all obtained on Silica Gel DF-5, the result of a single i.v. administration of tropital-methylene-^{14}C.

Fig. 2 illustrates the relative change in concentration with time of each metabolite detected in the autoradiograms (from 10.3 to 560 min after i.v. administration).

As depicted in Table I there are fourteen metabolites appearing in the bile containing ^{14}C either in the methylenedioxyphenyl moiety or transferred to other molecules.

Graph A of Fig. 2 shows that only four of these metabolites (R_F 0.68, 0.39, 0.35 and 0.17) appear at relatively high concentrations (spots at R_F 0.68 (piperonal) and

---

* Bile and urine samples were applied with a Radin-Pelids thin-layer sample streaker obtained from Applied Science Laboratories, State College, Pa., U.S.A.

** Obtained from Eastman Kodak Corp., Rochester, N.Y., U.S.A.

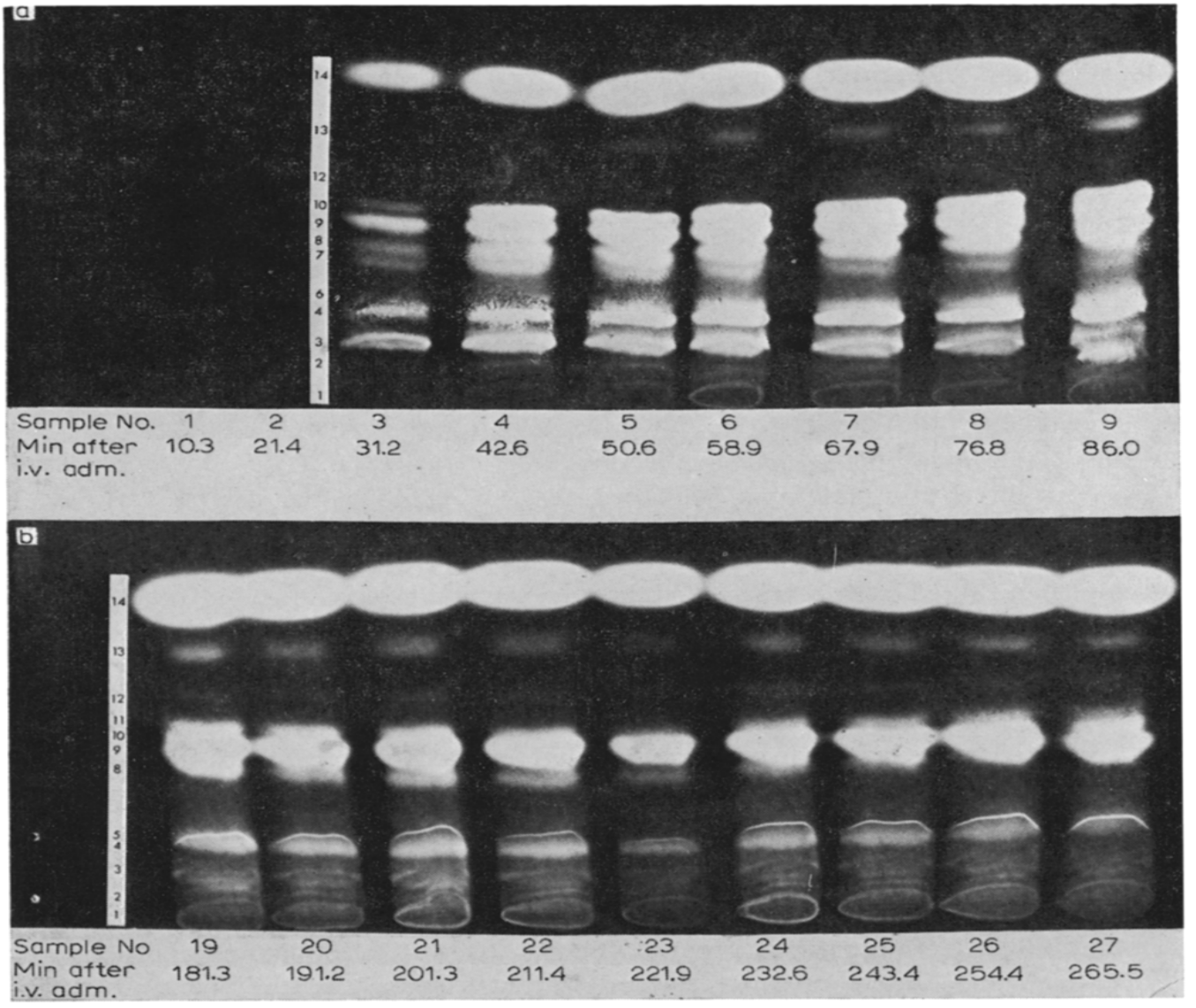


Fig. 1. Four-day autoradiogram of rat bile following i.v. administration of tropital-methylene-^{14}C. (a) Sample collection time 10.3–86.0 min. (b) Sample collection time 181.3–265.5 min.

R_F 0.39 accounting for 78 and 8.8 % respectively of the radioactivity in the bile)*. The remaining ten metabolites are present to much lower extent and with the metabolites at R_F 0.35 and 0.17 account for 13.2 % of the radioactivity in the bile.

Two metabolites (Nos. 5 and 11; R_F 0.19 and 0.40, Table I and Graph B of Fig. 2) appear later in the bile, at 50 and 162 min, respectively, after injection. The possibility exists that these as well as other radioactive metabolites could have been formed via prior methylene-^{14}C cleavage and secondary reaction.

It is difficult to account for the large number of metabolites unless the metabolites are conjugated or the tagged moiety combines with constituents of bile, blood, liver or kidneys components.

Ancillary studies were undertaken to examine both the purity and stability of

* As measured in a Packard Tri-Carb. Model 3365, scintillation counter, after removal of the spots from the developed chromatogram.

TABLE I

SUMMARY OF RADIOACTIVE METABOLITES PRODUCED IN BILE FROM RATS RESULTING FROM A SINGLE INTRAVENOUS ADMINISTRATION OF TROPITAL-METHYLENE-^{14}C

The data were obtained on Silica Gel DF-5 plates developed (30 min, 15 cm) with toluene–acetic acid–water (10:10:1). Plates were viewed under 2537 Å light before spraying and under visible light after spraying with chromotropic acid. Four-day autoradiograms were taken before spraying with acid.
Colors: B = blue; Bn = brown; R = red; V = violet.
Obs = obscured; and (—) = not detected. The bracket R_F values ( ) are those reported previously for tropital[1].

Metabolite	$R_F \times 100$	*Specific activity*	*Absorption maximum at 2537 Å*	*Chromotropic acid color development*	*First appearance of metabolite after injection (min)*	*Persistence of metabolite (min)*
1	0	+	obs.	obs.	31	560
2	8	+	obs.	—	31	560
3	12 (11)	+ +	+ + + +	Bn-R + + + +	31 (declines)	560
4	17	+ + +	+ +	Bn + +	31	379 (disappears)
5	19	+ +	—	Bn +	50	560
6	20	trace	—	—	31	122 (disappears)
7	26	+	—	—	31	86 (disappears)
8	30	+ +	—	V + +	31	212 (disappears)
9	35 (38)	+ + + +	B +	V + +	31	560
10	39	+ +	+	V + +	31 (declines)	560
11	40	+	—	—	162	560
12	45	trace	—	—	43	560
13	55	trace	trace	—	43	560
14	68 (72)	+ + + +	+ + +	V + +	31	560

commercial tropital by thin-layer chromatography. Tropital in acidic media is known to undergo degradation of the acetal side chain to piperonal. It is suggested that silica gel on thin-layer chromatoplates also leads to this degradation even when a non-polar developing solvent system such as hexane–ethyl ether (1:3) is employed.

Tropital dissolved in toluene–acetic acid–water (10:10:1) or acetic acid–butanol–water (1:4:5) and held at room temperature for 1 h, then developed with toluene–acetic acid–water (10:10:1) showed one spot at the same R_F as piperonal. This was further substantiated by comparing the U.V. absorption curve of the eluted sample. By spectrophotometric measurement at 311 mμ (maximum for piperonal, and minimum for tropital) it was found that tropital used in our studies contained 4–5 % piperonal. Purification on a silica gel plate by developing with hexane–ethyl ether (1:3) reduced the piperonal to about 2 %. Alumina, an alkaline adsorbent, reduced the amount of piperonal to about 1 %. We have been unable to purify tropital until completely free of piperonal.

Tropital has been found to contain three minor impurities other than piperonal.

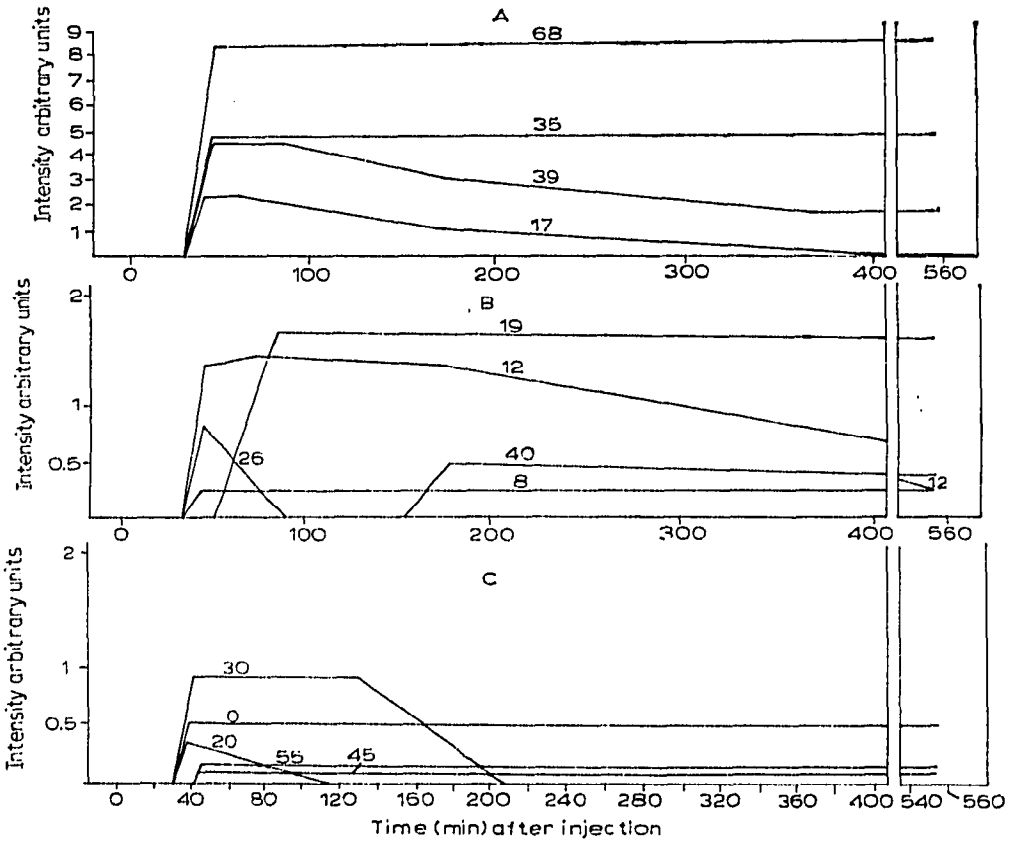


Fig. 2. Relative change in concentration with time of each metabolite detected in the autoradiograms. The numbers in graphs A, B and C indicate the R_F values × 100 given in Table I.

Their R_F values and some of their physical characteristics are described in Table II. Characterization of the above constituents is presently being undertaken by infrared spectroscopy.

Earlier studies utilizing non-radioactive tropital[1] revealed the presence of three spots in bile (R_F 0.11, 0.38 and 0.72) when toluene–acetic acid–water (10:10:1) was used as the developer. Two analogous spots in this study, R_F (× 100) 68 and 35,

TABLE II

TLC OF COMMERCIAL TROPITAL ON SILICA GEL DF-5 PLATES DEVELOPED WITH HEXANE–ETHYL ETHER (1:3)

R_F	*Comments*
0.00	Minor component–brown color in visible light. Orange fluorescence under 3660 Å. No characteristic U.V. absorption curve.
0.30	Minor component–U.V. absorption curve identical to tropital (230–320 mμ)
0.55	Piperonal–present between 4–5%. U.V. max. 272 and 311 mμ.
0.65	Tropital U.V. max. 285 mμ
0.72	Minor component–blue fluorescence under 3660 Å

were indicative of major excretion products in the bile (Fig. 2) and as indicated in earlier experiments were rapidly formed after compound administration and remained at approximately constant level for the duration of the experiment. The spot at R_F 68 in this study is piperonal which can result from tropital degradation in the presence of acid or by the action of liver enzymes.

TABLE III

SUMMARY OF METABOLITES PRODUCED IN URINE FROM RATS, RESULTING FROM A SINGLE I.V. ADMINISTRATION OF TROPITAL–METHYLENE-^{14}C

The data were obtained from chromatograms of rat urine on Silica Gel DF-5 plates developed with ethyl acetate–acetic acid–methanol (70:10:20). Photographic and autoradiogram procedures were the same as for bile.
Urine samples were collected from 67.9 to 553.2 min after tropital injection.

Metabolite R_F	*Maximum isotope activity*	*Maximum U.V. adsorption*	*Initial metabolite time after i.v. administration (min)*	*Metabolite duration time (min)*
0.00	+ +	+ +	211.4	315.4
0.48	+	+	211.4	341.8
0.72	+ + + +	+ + +	67.9	485.3
0.85	+	+	211.4	341.8

Table III depicts a composite summary of rat urine metabolites (R_F values, estimated isotope activity, U.V. adsorption intensity and time to first appearance of metabolite and this persistence) obtained on Silica Gel DF-5. As in the previously reported study[1] no free tropital was found in the urine. Only trace amounts of piperonylic acid (R_F 48) were found. The possibility of butyl carbitol (resulting from the degradation of the acetal side chain of tropital) being excreted in the urine as the corresponding glucoronide and/or sulfate conjugates exists, and is being currently elaborated utilizing glucuronidase and sulfatase.

Similarly, there is a possibility of non-radioactive metabolites, *e.g.*, 3,4-dihydroxy-benzaldehyde and/or 3,4-dihydroxy-benzoic acid being formed via opening of the methylenedioxy ring and the splitting of the acetal side chain of tropital and being excreted as a conjugate. This facet is similarly under investigation.

ACKNOWLEDGEMENT

This study was supported by Research Contract PH 43-64-57, National Cancer Institute, National Institutes of Health, Public Health Service and represents Paper No. 44 of this contract.

SUMMARY

Differences in rat bile and urine samples resulting from single intravenous administration of tropital-methylene-^{14}C were elaborated utilizing both autoradiography and thin-layer chromatography with toluene–acetic acid–water (10:10:1)

and ethyl acetate–acetic acid–methanol (70:10:20) as developing solvents. Detection was accomplished in the latter technique with chromotropic acid as well as a 2537 Å ultraviolet source.

REFERENCES

1 L. FISHBEIN, J. FAWKES, H. L. FALK AND S. THOMPSON, *J. Chromatog.*, 27 (1967) 153.
2 L. O. HOPKINS AND D. R. MACIVER, *Pyrethrum Post*, 8 (1965) 3.
3 M. BEROZA, *J. Agr. Food Chem.*, 11 (1963) 51.
4 P. KOTIN, H. L. FALK AND R. BUSSER, *J. Natl. Cancer Inst.*, 23 (1959) 541.