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Quantitation of [^{14}C]- and [^{3}H]pyridoxol by scintillation autography of thin layer chromatograms

During our study of the metabolism of [^{14}C]- and [^{3}H]pyridoxol, a need arose for the rapid identification and quantitation of its metabolites in body fluids after chromatography on cellulose thin layer plates. Normal procedures for localisation of metabolites could not be applied as in many instances a metabolite had to be recovered in pure form and in the small amounts present they could not be visualised by colour reactions. Also, as the radioactivity in some metabolites was very low, auto-radiography for their localisation proved to be a very lengthy process, in some cases necessitating exposures of over a month, or more for tritiated compounds. We therefore explored the process of scintillation autography as described by JOLCHINE[1]. We have compared the sensitivity of various different X-ray and photographic emulsions readily available in this country, and also investigated possible means of enhancing the sensitivity of the emulsion in order to reduce the exposure time.

Materials and methods

Radioactive samples. [^{14}C]Pyridoxol hydrochloride ([$^{14}C_2$]dicarbinol; 11 mC per mmole; Hoffman la Roche, Basle) and [^{3}H]pyridoxol (G) hydrochloride (64 mC per mmole; Radiochemical Centre, Amersham, Great Britain) were used as reference standards. A series of aqueous dilutions were prepared for chromatography.

Thin layer chromatography. Glass plates were coated with Whatman CC41 cellulose (H. Reeve Angel & Co. Ltd., London), prepared as follows: 20 g of cellulose were blended with 46 ml distilled water at the maximum speed of an M.S.E. homogenizer for 30 sec. The slurry was spread to a thickness of 0.25 mm on 20 × 20 cm glass plates. The plates were left to dry overnight at room temperature.

Standards of different concentrations were applied to the plates. The volume of each standard (1 μl) was kept constant in order to maintain a constant area of application. After equilibration (2 h) ascending chromatography was carried out at 20° in glass tanks using 1,4-dioxan–water (6:1, v/v) as the solvent system. After the solvent front had travelled 10 cm (50 min), the plates were removed and dried at room temperature. The R_F value for pyridoxol in the solvent system was 0.90.

Preparation of plates for scintillation autography. The dried TLC plates were sprayed twice with a scintillator mixture containing 4 g 2,5-diphenyloxazole (PPO) and 0.1 g 1,4-bis-2-(methyl-5-phenyloxazolyl)-benzene (dimethyl-POPOP) (scintillation grade, Packard Instr. Co., La Grange, Ill., U.S.A.) per litre of toluene. The solvent was blown off by air in a fume cupboard. The dried plates were ready for exposure to the films.

Exposure of films. Five Kodak films were investigated. X-ray emulsions; Kodirex, SO 288 (RP/S X-omatic medical), and Royal Blue X-ray films 'Estar'. Fast panchromatic negative emulsions: Panchro royal and Royal X-pan.

The films were placed in contact with the dried TLC plates and clamped between two glass plates. The sandwich was wrapped in aluminium foil and stored in the dark for 24 or 120 h. Operations involving the X-ray films were carried out under indirect

illumination using a Wratten 6B filter (exposure less than 2 min). The photographic emulsions were handled in darkness.

Development of films. After exposure, the films were rinsed in water, developed for 6 min in Kodak D-19b developer with occasional agitation, rinsed in water for 1 min, and fixed for 5 min in Kodak FX-40 X-ray fixer with HX-40 hardener added. After washing in water for a further 30 min, the films were hung up to dry. All procedures were carried out at 18–20°. Intermediate negatives were made by contact printing the developed films on Kodalith Ortho Type 3 film and developing for $2^3/_4$ min in Kodalith super developer.

Effect of reflectors, temperature, scintillator saturation, and single emulsion development. (a) TLC plates were obtained as above. After spraying with scintillator, plates with and without aluminium foil backings were exposed to the films at room temperature and at −5°. (b) TLC plates were obtained as above but the plates were saturated with the scintillator mixture and sealed in flush fitting polythene bags to prevent evaporation of the toluene solution. (c) After exposure, the double-sided X-ray films were attached to glass plates by waterproof adhesive tape. Development of the films was carried out as described above. The films were separated from their backing plates before the fixing stage.

Recovery of activity deposited on plates. The areas on the TLC plates corresponding to the images on the films were sucked by a small vacuum pump into Pasteur pipettes fitted with pledgets of glass wool. The cellulose in each pipette was shaken out into a centrifuge tube and the pipette washed out with 5 ml methanol. After agitating the mixture on a "buzzer" type of mixer, the tubes were centrifuged. 4 ml aliquots of the supernatants were added to vials containing 10 ml scintillator mixtures identical with that used for spraying the plates. The vials were counted in a Packard TriCarb Spectrometer. Counts were converted to d.p.m. after appropriate quench correction, and recorded as mμC recovered.

Results and discussion

The limits of detection for ^{14}C and ^{3}H by scintillation autography are shown for the different films in Table I. The most sensitive films were Royal Blue for ^{14}C and SO 288 for ^{3}H (Figs. 1–4). This was expected as Royal Blue and SO 288 are 'screen'

TABLE I

VISUAL LIMITS OF DETECTION OF [^{14}C]- AND [^{3}H]PYRIDOXOL ON VARIOUS FILMS BEFORE AND AFTER CHROMATOGRAPHY EXPRESSED AS mμC APPLIED TO PLATES

Area of spot after chromatographic run approximately 1 cm^2.

Isotope	*Exposure time (h)*	*Kodirex*	*SO 288*	*Royal Blue*	*Panchro royal*	*Royal-X pan*
^{14}C before run	24	<0.1	0.1	0.05	0.2	0.1
after run	24	<0.5	<0.5	0.05	—	—
after run	120	0.05	0.05	<0.05	—	—
^{3}H before run	24	250	25	25	100	50
after run	24	500	25	25–50	—	—
after run	120		5	5–10	—	—

Fig. 1

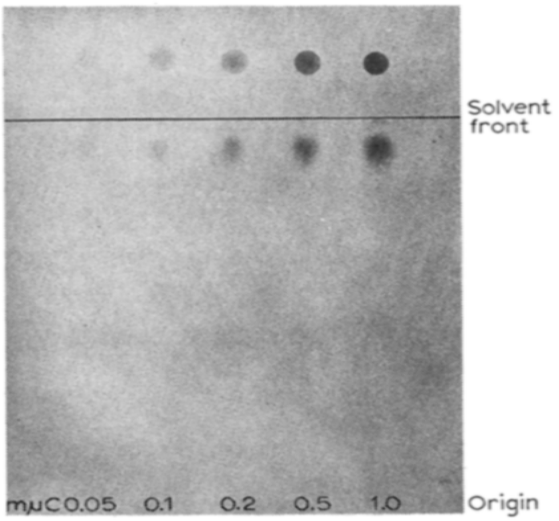

Fig. 2

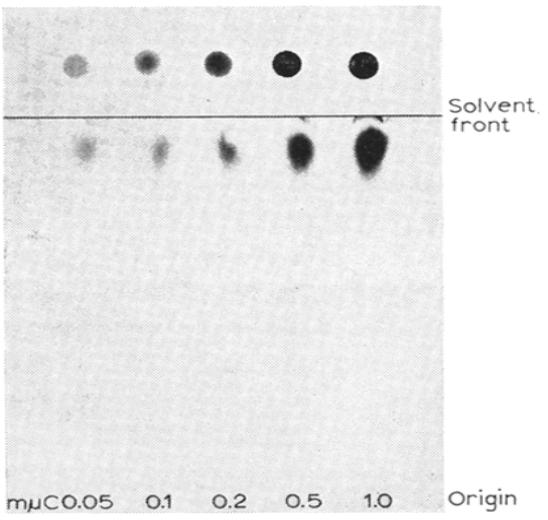

Fig. 3

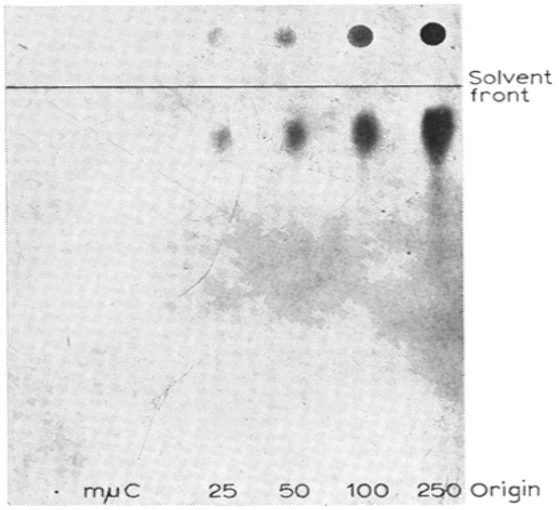

Fig. 4

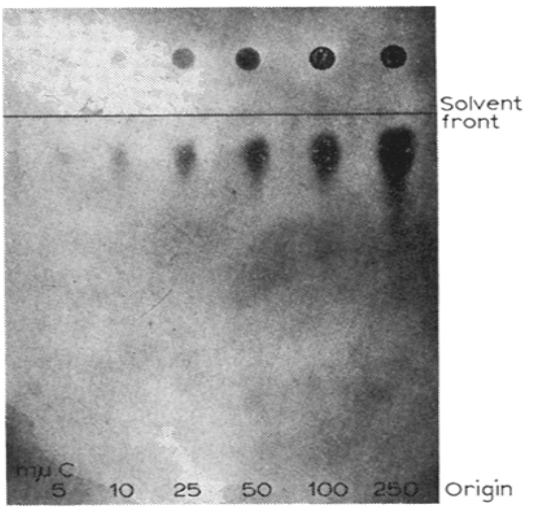

Fig. 1. Print of a Kodalith negative prepared from a Royal Blue film after a 24-h exposure to [^{14}C]pyridoxol. The top row of images shows the unchromatographed samples.

Fig. 2. Print of a Kodalith negative prepared from a Royal Blue film after a 120-h exposure to [^{14}C]pyridoxol. The top row of images shows the unchromatographed samples.

Fig. 3. Print of a Kodalith negative prepared from a SO 288 film after a 24-h exposure to [^{3}H]-pyridoxol. The top row of images shows the unchromatographed samples.

Fig. 4. Print of a Kodalith negative prepared from a SO 288 film after a 120-h exposure to [^{3}H]-pyridoxol. The top row of images shows the unchromatographed samples.

type X-ray films sensitive to fluorescent light, whilst Kodirex, a 'non-screen' type film, is mainly sensitive to radiation.

Aluminium foil reflectors and exposure at $-5°$ had no apparent effect on the sensitivity of detection. Saturation with scintillator solution reduced the sensitivity. One-sided development of the X-ray films reduced the background considerably thus increasing the contrast and hence the apparent sensitivity of detection.

TABLE II

RECOVERY OF [^{14}C]- AND [^{3}H]PYRIDOXOL APPLIED TO PLATES BEFORE AND AFTER CHROMATOGRAPHIC RUNS

Isotope	*mμC added to plate*	*Before run*		*After run*	
		mμC recovered	*% recovery*	*mμC recovered*	*% recovery*
^{14}C	0.10	0.095	95	0.093	93
	0.20	0.209	104	0.180	90
	0.40	0.384	96	0.368	92
	0.80	0.79	99	0.710	89
^{3}H	50	44.2	88	49.8	100
	100	98.8	99	96.5	96.5
	250	248	99	218	88
	500	555	111	442	88

If necessary, the sensitivity can be increased a further two-fold by preparing an intermediate Kodalith negative as described before.

Recoveries of radioactivity from the plates after localisation are shown in Table II. They ranged from 88–111 %.

Though the optimum sensitivity of detection for tritium is the same as that reported by JOLCHINE, our method has increased the sensitivity to carbon-14 by ten to twenty times.

For carbon-14 compounds the lowest limit of detection by scintillation autography is 0.05 mμC for a 24-h exposure period. This level of radioactivity would not be detected by autoradiography as the time of exposure would run to over a month by which time aerial and chemical fogging of the emulsion would take place and the image would be indiscernible as it would merge with the background.

In the case of tritium compounds only a ten-fold decrease in the exposure time can be achieved by autoscintillography over autoradiography.

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1 G. JOLCHINE, *Physiol. Vegetale*, 2 (1964) 341.

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