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Note

Rapid method for the purification of [3,5-^{14}C]paracetamol

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Paracetamol is a widely used analgesic which is generally well tolerated at the therapeutic dose but causes centrilobular hepatic necrosis when taken in overdose [1]. Various mechanisms for the toxicity of paracetamol have been proposed, but covalent binding of an active metabolite to vital cellular proteins [2] is widely accepted as an essential step.

In many of the studies concerned with mechanism of injury, covalent binding of [^{14}C]paracetamol to hepatocellular proteins has been measured. We have also carried out such studies, but it was found that the commercially available [^{14}C]paracetamol, of apparently high purity, as indicated by thin-layer chromatography (TLC), contained an impurity that bound strongly to proteins.

Here we report a simple and rapid high-performance liquid chromatographic (HPLC) technique for the purification to $\geqslant$99.9% of commercially available [^{14}C]paracetamol on a preparative scale using an ordinary analytical column. By this technique, a ^{14}C-labelled contaminating compound (approximately 1.5% of total preparation) which was not detected by any of the TLC techniques employed in previous work [2,3] and which bound covalently to albumin, without prior need for metabolic activation, was isolated.

MATERIALS AND METHODS

[^{14}C]Paracetamol, labelled in the ring C-3 and C-5 positions (specific activity 35 mCi/mmol) was obtained from Amersham International (Amersham, Great Britain). TLC silica gel plates, containing a fluorescent indicator F-254, were supplied by E. Merck (Darmstadt, G.F.R.). All solvents used for TLC techniques were of AnalaR grade and obtained from BDH (Poole, Great Britain). HPLC grade methanol was from Fisons (Loughborough, Great Britain) and Hypersil ODS 5 μm HPLC column packing was purchased from Magnus Scien-

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tific (Sandbach, Great Britain). Instagel scintillation cocktail was supplied by Packard (Caversham, Great Britain) and crystalline paracetamol and bovine serum albumin (BSA) were purchased from Sigma (London) (Poole, Great Britain).

TLC of [^{14}C] paracetamol

TLC analysis was carried out on silica gel 60 plates. Chromatographic separations, employing 5 μl (45 μg) of 60 m*M* paracetamol stock solution as standard, were carried out in vapour-saturated glass chromatography tanks using one of the following solvent systems: System 1: propan-2-ol–acetone–ammonia solution (Sp. gr. 0.88)–water (70:20:20:20, v/v); System 2: ethyl acetate [2, 3]; System 3: ethyl acetate—methanol—glacial acetic acid—water (12:6:0.2:1.8, v/v) [2, 3].

Following chromatographic development for 1—3 h, the plates were allowed to dry and then observed under UV light at 254 nm. Strips (1 cm) were scraped off, from the line of origin up to the solvent front, and each fraction suspended as a fine slurry in 1.5 ml water. After addition of 5.0 ml Instagel, the fractions were counted in Packard Tri-Carb liquid scintillation counter. Counts were converted to disintegrations per minute (dpm) using the automatic external standard, calibrated with an internal [^{14}C] hexadecane standard. The silica powder suspended in water did not interfere with the estimation of paracetamol radioactivity.

HPLC of [^{14}C] paracetamol

A Waters Assoc. (Milford, MA, U.S.A.) Model 6000A constant flow chromatography pump connected to a Waters Assoc. universal liquid chromatography injector, with a 2.0-ml back-fill loop, was used as the solvent delivery system. This led to a column, 25 cm × 4 mm I.D. packed with Hypersil ODS 5 μm.

Chromatographic purification of [^{14}C] paracetamol was carried out using a methanol–water (10:90, v/v) solvent system pumped at a constant flow-rate of 1.0 ml/min. The column was loaded by injection of 1.0 ml of paracetamol solution in water containing approximately 1.0 mg and about 15 μCi. Prior to the chromatographic run, this comparatively large quantity of paracetamol was concentrated down onto the column, by pumping a 100% water mobile phase, instead of methanol—water (10:90), for 2 min. During the chromatographic operation, up to 60 fractions of 0.5 ml were collected, using a LKB 2112 Redirac fraction collector.

Standardisation of the chromatographic separation was achieved using unlabelled paracetamol. The fractions collected were analysed for optical absorption at 243 nm wavelength, in a Pye-Unicam SP 30 spectrophotometer.

Prior to recovery of the radioactive paracetamol, 20-μl aliquots of each fraction were analysed for radioactivity. The seven fractions (from 19 to 25) containing the highest counts of the major peak were pooled, saturated with sodium chloride and gently mixed with re-distilled diethyl ether. Purified [^{14}C] paracetamol, which had been back-extracted into the solvent ether phase, was crystallised out by evaporating the diethyl ether to dryness under a gentle stream of oxygen-free nitrogen, and finally dissolved in water as a concentrated working solution.

The radioactivity profile of the fractions collected revealed a contaminating ^{14}C-labelled compound 2, which was also purified and recovered (fractions 34 to 43).

Between runs the column was cleaned by pumping 100% methanol through the system. This led to recovery of a very small fraction (0.5% of total) of radioactive material.

Covalent binding study

Preliminary experiments to study non-enzyme catalysed covalent binding of both [^{14}C]paracetamol and the ^{14}C-labelled contaminant (compound 2) were carried out using either a Hepes—Ringer buffer system [4], containing 2.5% bovine serum albumin, or an aqueous suspension of rat liver hepatocytes which had been prepared by a modification of the method of Seglen [5]. For the purposes of this study the hepatocytes were made non-viable, by suspension and vigorous mixing in water.

Flasks containing 2.5-ml final volumes of incubation medium containing albumin or the approximately 300 mg/ml cell suspension, plus either purified radioactive paracetamol (approx. 0.5 μCi, and 25 μmol) or compound 2 (approx. 0.12 μCi and 3.4 nmol, if the specific activity is that of the original [^{14}C]paracetamol as bought) were incubated at 37°C in a shaking water bath. Aliquots (0.5 ml) were withdrawn from the flasks at 0, 1, 2 and 4 h of incubation, and were analysed for total covalently bound ^{14}C-labelled material to protein. The cellular preparation was precipitated with 10% (w/v) trichloroacetic acid (TCA) in water and washed four times with the TCA and then twice with methanol—water (80:20, v/v). Each washing stage was carried out by suspension of the protein pellet followed by centrifugation at 1500 *g* for 3 min.

The albumin precipitates were also washed as described for the protein pellets, except that at the methanol washing stages, the precipitates were initially dissolved in 5.0 ml of methanol—water (80:20) followed by re-precipitation with 5.0 ml of 10% TCA.

The final washed protein pellets were suspended in 2.0 ml 1 *M* sodium hydroxide solution and digested overnight at 45°C. Aliquots (1 ml) of the digests were suspended in 5.0 ml. Instagel, neutralised with 1.0 ml 1 *M* hydrochloric acid and subjected to liquid scintillation counting.

Protein was also estimated by the method of Lowry et al. [6], in the alkaline digests.

RESULTS

TLC analysis

All TLC separations, irrespective of the development system used, displayed a single major [^{14}C]paracetamol spot, which corresponded to the reference sample spot in R_F and fluorescence quenching when viewed under the UV light at 254 nm. The R_F values and percent recovery of total radioactivity for each system are as shown in Table I.

Radioactivity on the TLC plates corresponded, in all cases, with the major spot visualised by its UV fluorescence quenching.

TABLE I

R_F VALUE OF AND PERCENT RADIOACTIVITY RECOVERY FROM [^{14}C]PARACETAMOL SPOT VISUALISED IN DIFFERENT SOLVENT SYSTEMS

Chromatographic methods and solvents are as described in the Materials and methods section. A 5-μl volume of [^{14}C]paracetamol solution containing 136,000 dpm was loaded onto the TLC plates. A 1.5% impurity spot containing about 2000 dpm, an easily countable amount, could not be detected. TLC of the isolated compound 2 also showed that the R_F value of this compound was very similar to that of paracetamol and *p*-aminophenol (PAP) in the three TLC systems chosen. A further TLC system [7] could distinguish paracetamol, PAP and the impurity, compound 2. PAP and compound 2 both gave a long tailing spot (R_F 0.18—0.35), that overlapped the distinct paracetamol spot (R_F 0.24). However, this does not suffice to give a distinct spot for a 1.5% impurity of compound 2 in paracetamol.

Solvent system	R_F	Percent of total activity
1	0.74	99.7
2	0.29	99.6
3	0.80	99.4

HPLC analysis

Analysis, by absorption spectroscopy, of the fractions collected after loading 1.0 mg of non-radioactive paracetamol indicated that the retention time of paracetamol on the column under the specified conditions was 9—10 min and only one peak came off the column.

The radioactivity profile of the fractions collected after loading 1.0 mg of [^{14}C]paracetamol displayed two major peaks, of which the first peak had both a retention time and a profile equivalent to the absorption profile of authentic paracetamol (Fig. 1). The second peak had a longer retention time of 16—18 min and did not show up on the UV absorption monitor set at 243 nm (Fig. 1).

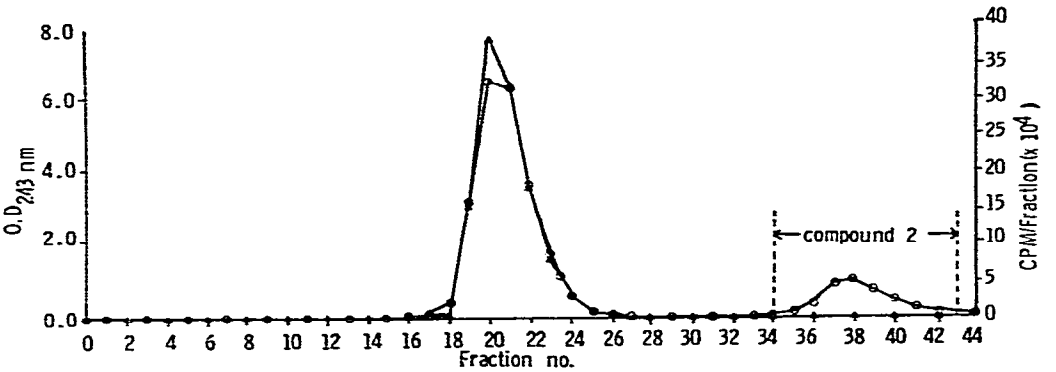

Fig. 1. Spectrophotometric absorption profile (▲) and radioactivity profile (○) of the fractions collected after separate HPLC runs are shown. Unlabelled paracetamol (approx. 1.0 mg) was chromatographed and the absorbance profile was determined for each 0.5-ml fraction. The radioactivity profile was determined by counting 20-μl aliquots of each fraction. For compound 2 only, the cpm/fraction is calculated as a multiplier factor of 10^3 instead of 10^4.

Calculation of total radioactive counts from each HPLC run indicated that the [^{14}C]paracetamol accounted for approximately 98.5% and the ^{14}C-labelled compound 2 accounted for about 1.5% of the total counts recovered.

When the separated paracetamol was re-run only one peak was found, indicating that peak 2 was not a breakdown product in equilibrium with paracetamol. Similarly when peak 2 material was re-run, it re-appeared at the same retention time. Preliminary observations of the ^{14}C-labelled compound 2 material suggest that this compound may be *p*-aminophenol (PAP), which is used in the preparation of [^{14}C]paracetamol. Like PAP it forms a dark brown compound on standing in air.

Non-enzyme catalysed covalent binding

It was noticed that very little purified [^{14}C]paracetamol bound covalently to albumin in the Hepes—Ringer buffer. In contrast, the binding of the hepatocellular proteins was slightly elevated initially and then quite markedly by the end of the 4-h incubation period (Table II).

The ^{14}C-labelled contaminating compound 2 on the other hand bound extensively, in a cumulative fashion to both albumin and hepatocellular protein, in spite of being present at far lower concentration.

TABLE II

NON-ENZYME CATALYSED COVALENT BINDING OF PURIFIED [^{14}C]PARACETAMOL AND [^{14}C]COMPOUND 2 TO PROTEIN

Approximately $1.3 \cdot 10^6$ dpm of [^{14}C]paracetamol was added to flasks 1 and 3, and $0.3 \cdot 10^6$ dpm of [^{14}C]compound 2 was added to flasks 2 and 4. Samples were taken, washed and analysed as described in the Materials and methods section.

Incubation mix	Incubation time (h)	dpm bound/ flask	dpm bound/ mg protein	dpm bound/ 10^3 dpm added
(1) Purified	0	24.8	0.7	0.02
[^{14}C]paracetamol	1	99.4	3.5	0.08
Hepes—Ringer	2	125.8	3.5	0.10
albumin	4	176.1	3.9	0.14
(2) Purified	0	12.4	0.4	0.04
[^{14}C]compound 2	1	1125.0	33.5	3.75
Hepes—Ringer	2	1543.2	47.6	5.14
albumin	4	2780.0	60.4	9.27
(3) Purified	0	49.7	3.0	0.04
[^{14}C]paracetamol	1	600.0	36.6	0.46
Hepatocytes	2	1321.0	94.4	1.02
in water	4	4762.5	270.6	3.66
(4) Purified	0	87.5	4.8	0.29
[^{14}C]compound 2	1	3879.0	262.1	12.93
Hepatocytes	2	8773.2	577.2	29.24
in water	4	20037.5	1138.5	66.79

DISCUSSION

The drug paracetamol shows a combination of usefulness in normal dose and risk of injury in overdose that makes it imperative to understand its mechanism of toxicity. In order that this goal be achieved, it is also important that the compound under study is both authentic and of highest purity available so that mis-interpretation of experimental results can be avoided.

The covalent binding studies described here show that the purified [^{14}C] paracetamol used does not bind to albumin to any significant extent. However, the binding of [^{14}C] paracetamol to cellular proteins may be due to the presence of endogenous haem and co-factors even in the non-viable cell preparation.

The contaminating ^{14}C-labelled compound 2 binds to both albumin and cellular proteins far more rapidly and extensively than [^{14}C] paracetamol, even though present at only a quarter of the activity of the paracetamol. This again emphasises the need for working with compounds of both very high radiochemical and chemical purities.

In some previous studies where covalent binding of paracetamol was thought to be the cause of paracetamol hepatotoxicity, radioisotope preparations of apparent radiochemical purities of $\geqslant$99.9%, as assessed by TLC techniques, were used. Results presented in the present study clearly show that TLC techniques, as a check for radiochemical purity, may not be enough and that other methods of much higher resolution may have to be employed for this purpose. The HPLC technique described is suitable since it incorporates the high sensitivity required for analytical work. In addition, the technique is suitable as a preparative method, because of its simplicity, rapidity and the relative ease with which very highly purified preparations of [^{14}C] paracetamol may be obtained.

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REFERENCES

1 O. James, M. Lesna, S.H. Roberts, L. Pulmon, A.P. Douglas, P.A. Smith and A.J. Watson, Lancet, ii (1975) 579.
2 J.R. Mitchell, D.J. Jollow, W.Z. Potter, D.C. Davis, J.R. Gillette and B.B. Brodie, J. Pharmacol. Exp. Ther., 187 (1973) 185.
3 W.Z. Potter, D.C. Davis, J.R. Mitchell, D.J. Jollow, J.R. Gillette and B.B. Brodie, J. Pharmacol. Exp. Ther., 187 (1973) 203.
4 A.E.M. McLean and L. Nuttal, Biochem. Pharmacol., 27 (1978) 425.
5 P.O. Seglen, Methods Cell Biol., 13 (1976) 29.
6 O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.J. Randall, J. Biol. Chem., 193 (1951) 265.
7 R.A. Savidge and J.S. Wragg, J. Pharm. Pharmacol., 17 (1965) 60S.