Journal of Chromatography, 174 (1979) 165–175

CHROM. 11,781

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR ACETAMINOPHEN AND PHENACETIN IN THE PRESENCE OF THEIR METABOLITES IN BIOLOGICAL FLUIDS

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(First received September 15th, 1978; revised manuscript received February 6th, 1979)

SUMMARY

We propose a method in which tracer amounts of a radiolabeled compound are used as the internal standard for the same unlabeled compound in high-performance liquid chromatography. The approach is valuable when a response from the internal standard becomes undesirable due to the presence of interference by the metabolites. We tested our approach with phenacetin and its metabolites, acetaminophen, 2-hydroxyphenacetin, N-hydroxyphenacetin, phenetidine, acetaminophen sulfate conjugate and acetaminophen glucuronide conjugate in biological fluids with the use of [^{14}C]phenacetin and [^{3}H]acetaminophen as the internal standards, and were able to quantitate both phenacetin and acetaminophen simultaneously. We also tested the alternative approach in which the unlabeled drug was used as internal standard for tracer amounts of the same radiolabeled compound, with phenacetin and acetaminophen as the internal standards for tracer amounts of [^{14}C]phenacetin and [^{3}H]acetaminophen. Again, we were able to quantitate the two tracer radiolabeled compounds simultaneously.

INTRODUCTION

The advent of high-performance liquid chromatography (HPLC) has become a useful research tool for the quantitation of many compounds. Since the detection by HPLC is by the absorbance (or fluorescence) of a compound, compounds with similar structural aspects will interact similarly to a given solvent system, and may have similar retention times. When the extraction properties and the absorbance (or fluorescence) of a drug and its metabolites are similar, identification of a drug in the presence of its metabolites becomes increasingly more difficult. The presence of the

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metabolites, therefore, may complicate the identification of the parent drug, and the probability of interference will increase proportionally with the number of metabolites, especially when an internal standard is incorporated into the method. It is also desirable to account for losses of the drug during the extraction of a biological sample, but there is little assurance that the recovery of the usual kind of internal standard will be exactly the same as the recovery of the drug.

The contribution of a response (peak) by an internal standard is sometimes an undesirable aspect in quantitation. When a tracer amount of a radiolabeled compound is used as an internal standard for the unlabeled compound, however, the internal standard will remain "invisible" but yet can be quantitated by measuring the radioactivity.

We developed a method for the assay of phenacetin and its metabolite, acetaminophen (paracetamol), in the presence of other metabolites of phenacetin in which [^{14}C]phenacetin and [^{3}H]acetaminophen are used as internal standards for the unlabeled drugs. We also describe a method in which unlabeled phenacetin or acetaminophen is used as the internal standard for tracer amounts of the radiolabeled drugs. We tested this method with varying tracer amounts of radiolabeled [^{14}C]phenacetin and [^{3}H]acetaminophen with unlabeled phenacetin and acetaminophen as the internal standards, respectively.

MATERIALS AND METHODS

Phenacetin and acetaminophen, obtained from Eastman-Kodak (Rochester, N.Y., U.S.A.) were recrystallized in methanol, and their purities were verified by melting point determinations, mass spectroscopy and thin-layer chromatography (TLC). Further examination by HPLC revealed a single peak when the compounds were analyzed.

General tritium ring-labeled acetaminophen (specific activity 937.5 mCi/mmole; New England Nuclear, Boston, Mass., U.S.A.) and [carbonyl-^{14}C]phenacetin (specific activity 55.7 mCi/mmole, synthesized by a previously described method[1]) were purified by TLC on silica gel GF (250 μm) thin-layer plates (Analtech, Newark, Del., U.S.A.) with a solvent system of diethyl ether. The purity of each compound was verified by scanning the plates with a radioactivity chromatogram scanner (Model 7201; Packard, Downers Grove, Ill., U.S.A.). Only one radioactive fraction was observed and it had the same R_F as the authentic unlabeled standard.

Two metabolites of phenacetin, phenetidine and 2-hydroxyphenacetin, were obtained from Eastman-Kodak; N-hydroxyphenacetin was synthesized by a previously described method[2]. The metabolites of acetaminophen, acetaminophen sulfate conjugate and acetaminophen glucuronide conjugate, were gifts obtained from McNeil Labs. (Fort Washington, Pa., U.S.A.).

All solvents used were of spectrograde quality; methanol and ethyl acetate were obtained from Matheson, Coleman & Bell (Houston, Texas, U.S.A.), and acetonitrile was obtained from Fisher Scientific (Pittsburgh, Pa., U.S.A.). The PIC B-7 reagent, a heptanesulfonic acid in 0.05 *M* acetic acid solution, was obtained from Waters Assoc. (Milford, Mass., U.S.A.). A solvent, acetonitrile–water–PIC B-7 (30:70:1.6, v/v/v) was previously filtered through a millipore filter (Millipore, Bedford, Mass., U.S.A.) under vacuum prior to use.

Methanolic solutions of acetaminophen (200 mg/l) and phenacetin (500 mg/l) were used as stock solutions; a 1:10 dilution of these stock solutions were prepared with methanol. Methanolic solutions of phenetidine (223 mg/l), 2-hydroxyphenacetin (300 mg/l) and N-hydroxyphenacetin (550 mg/l), metabolites of phenacetin[3–5], were also prepared. An aqueous solution containing a mixture of [^{3}H]acetaminophen (23×10^6 dpm/ml) and [^{14}C]phenacetin (6.8×10^6 dpm/ml) was used as internal standards in the assay of unlabeled acetaminophen and phenacetin. A methanolic solution containing a mixture of unlabeled acetaminophen (100 mg/l) and phenacetin (250 mg/l) was used as a stock solution for the internal standards in the assay of tracer amounts of radiolabeled acetaminophen and phenacetin.

Extraction of unlabeled acetaminophen and phenacetin in blood

Varying amounts of purified unlabeled acetaminophen (0.5–20 μg) and phenacetin (2.5–500 μg) in methanolic solutions were added to a 125 × 10 mm culture tube, and the methanol was evaporated to dryness. Aliquots of 0.1 ml rat blood (previously stored in EDTA vacutainer; Becton-Dickinson, Rutherford, N.J., U.S.A.), 0.1 ml of the aqueous solution containing the radiolabeled internal standards, 1.0 ml of 0.1 *M* acetate buffer pH 5.3 and 6 ml ethyl acetate were added to the tubes. After capping the tubes with PTFE-sealed caps, the tubes were shaken by a tilt-type mixer (Ames, Elkhart, Ind., U.S.A.) for 15 min, and then centrifuged for 10 min. A 5-ml aliquot of ethyl acetate was transferred into a 3-dram glass vial and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100–300 μl methanol, and 10 μl were injected into a high-pressure liquid chromatograph.

Extraction of tracer amounts of radiolabeled acetaminophen and phenacetin

Various tracer amounts of purified radiolabeled [^{3}H]acetaminophen (36×10^3–5930×10^3 dpm) and [^{14}C]phenacetin (1.6×10^3–879×10^3 dpm) were added to a 125 × 10 mm culture tube. Aliquots of 0.1 ml rat blood, 0.1 ml methanolic solution containing unlabeled acetaminophen (10 μg) and phenacetin (25 μg), 1.0 ml of 0.1 *M* acetate buffer, pH 5.3, and 6.0 ml ethyl acetate were added. The tubes were shaken and centrifuged as described above. A 5-ml volume ethyl acetate was transferred into a 3-dram glass vial and evaporated to dryness under a stream of nitrogen. The residue was dissolved in 100 μl methanol, and 10 μl were injected into a high-pressure liquid chromatograph.

HPLC

HPLC was performed with a Waters Assoc. chromatograph (Model 6000 A) that was equipped with a dual-wavelength UV absorbance detector (254 and 280 nm) and a μBondapak C_{18} column (30 cm × 4 mm I.D., 10 μm; Waters Assoc.). A mobile phase of acetonitrile–water–PIC B-7 (30:70:1.6, v/v/v) was utilized at a flow-rate of 1 ml/min. The samples were injected onto the column with a Model U6K injector (Waters Assoc.) and the chromatograms were recorded at wavelengths of 254 and 280 nm with an OMNI-scribe TM dual-pen recorder (Houston Instruments, Houston, Texas, U.S.A.).

Sample collection

An extracted sample containing unlabeled acetaminophen (10 μg) and phen-

acetin (25 μg), [^{3}H]acetaminophen and [^{14}C]phenacetin was injected into the liquid chromatograph, and the eluted solvent was collected into 20-ml glass scintillation vials at different time intervals. After the addition of 17 ml Aquasol (New England Nuclear), the radioactivity of each sample was counted by a scintillation counter (Searle, Model 6880: Nuclear Chicago, Chicago, Ill., U.S.A.) with a dual-channel program which monitored both ^{3}H- and ^{14}C-counts.

Solvent elutions at the post injection intervals 3.5–5.0 min and 8.5–10.5 min were collected; these time intervals were based on the correspondence of the radioactive counts with the chromatograms. The injection port was flushed with solvent between injections to avoid contamination between samples.

Interferences

Possible interferences by the solvent, methanol and substances in rat blood were tested by injection of 10 μl of methanol, and 10 μl of an extracted blank sample containing rat blood into the liquid chromatograph. Interference due to acetaminophen sulfate conjugate and acetaminophen glucuronide conjugate was tested by injection of the compounds into the liquid chromatograph in the absence and in the presence of acetaminophen and phenacetin. Interference by phenetidine, 2-hydroxyphenacetin and N-hydroxyphenacetin was tested by adding 22.3 μg phenetidine, 30 μg 2-hydroxyphenacetin, 55 μg N-hydroxyphenacetin, 10 μg acetaminophen and 25 μg phenacetin to blood and subjecting them to the assay procedure; 10 μl of the methanol extract were injected into the liquid chromatograph.

Specificity of the assay

The specificity of the assay for acetaminophen and phenacetin was confirmed by collecting the fraction at 3.5–5.0 min and 8.5–10.5 min from a sample containing acetaminophen, phenacetin, 2-hydroxyphenacetin, N-hydroxyphenacetin and phenetidine, and analyzing them by mass spectroscopy. The analyses were performed by direct insertion probe on a V.G. Micromass 16F mass spectrometer at an accelerating voltage of 4 kV, an electron energy of 70 eV, an ionizing current of 100 μA and a source temperature of 200°. The fractions were also spotted on silica gel GF thin-layer plates and developed with a solvent system of diethyl ether. The regions of radioactivity as detected by a radiochromatogram scanner (Packard, Model 7201) co-chromatographed with the authentic standards added (detected under ultraviolet light). The R_F values were: phenacetin, 0.32; acetaminophen, 0.20; 2-hydroxyphenacetin, 0.46; N-hydroxyphenacetin, 0.12; phenetidine, 0.60; acetaminophen sulfate and glucuronide conjugates stayed at the origin.

RESULTS

The chromatograms of both methanol and the blank blood sample (Fig. 1) at the highest sensitivity employed [0.05 absorbance unit (a.u.) at 254 nm and 0.02 a.u. at 280 nm] showed the absence of interference to the chromatograms from an extracted sample containing 10 μg of unlabeled acetaminophen and 25 μg of phenacetin at both wavelengths (Fig. 2A). The retention times of acetaminophen and phenacetin were 4 and 9 min, respectively, and were constant from day to day.

The regions of radioactivity contained in the eluted solvent corresponded

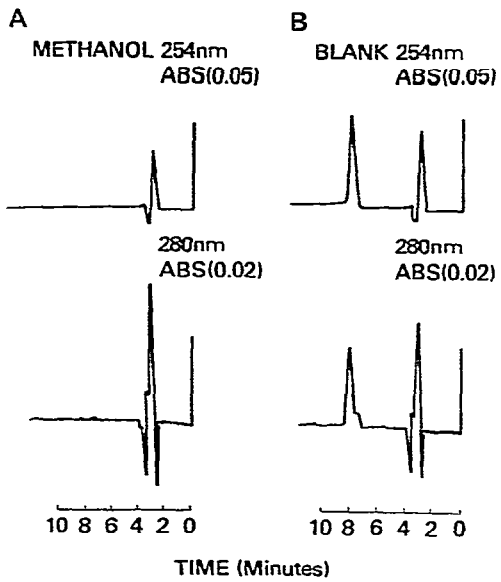


Fig. 1. The chromatograms of reagent blanks. Absorbance was measured at 254 nm and 280 nm. (A) Chromatogram of methanol alone; (B) chromatogram of a methanol solution of the ethyl acetate extract of blank blood. The values in parentheses indicate the sensitivity range of the UV detector.

with the peaks detected at both wavelengths, with a time-lag of 0.2 min between sample detection and sample collection (Fig. 2B). Recovery of the radioactive counts eluted from the column between 3.5 and 5.0 min for [^{3}H]acetaminophen and 8.5 and 10.5 min for [^{14}C]phenacetin was complete when compared to the counts contained in the same volume of an uninjected sample. When acetaminophen sulfate conjugate and acetaminophen glucuronide conjugate in aqueous solutions were injected into the liquid chromatograph the compounds eluted with retention times of less than 3.5 min. Furthermore, these polar conjugates were not extracted into ethyl acetate. When an extracted sample containing phenetidine (22.3 μg), 2-hydroxyphenacetin (30 μg), N-hydroxyphenacetin (55 μg), acetaminophen (10 μg) and phenacetin (25 μg) was injected into the liquid chromatograph the retention times of phenetidine and 2-hydroxyphenacetin were 5.8 and 7.3 min, respectively. N-Hydroxyphenacetin was not eluted from the column (Fig. 3).

Specificity

The specificity of the assay procedure was confirmed by the constancy of the ratio of the peak heights of the chromatograms at 254 nm and at 280 nm for both acetaminophen and phenacetin even in the presence of the metabolites, 2-hydroxyphenacetin, N-hydroxyphenacetin and phenetidine. Furthermore, TLC of the fractions indicated the presence of only one radioactive peak that co-chromatographed with either authentic acetaminophen or phenacetin but not with the metabolites of phenacetin. Mass spectral analyses of the collected fractions confirmed that only phenacetin and acetaminophen were present.

Unlabeled acetaminophen and phenacetin

The peak height of a sample was calculated as the height of the chromatogram

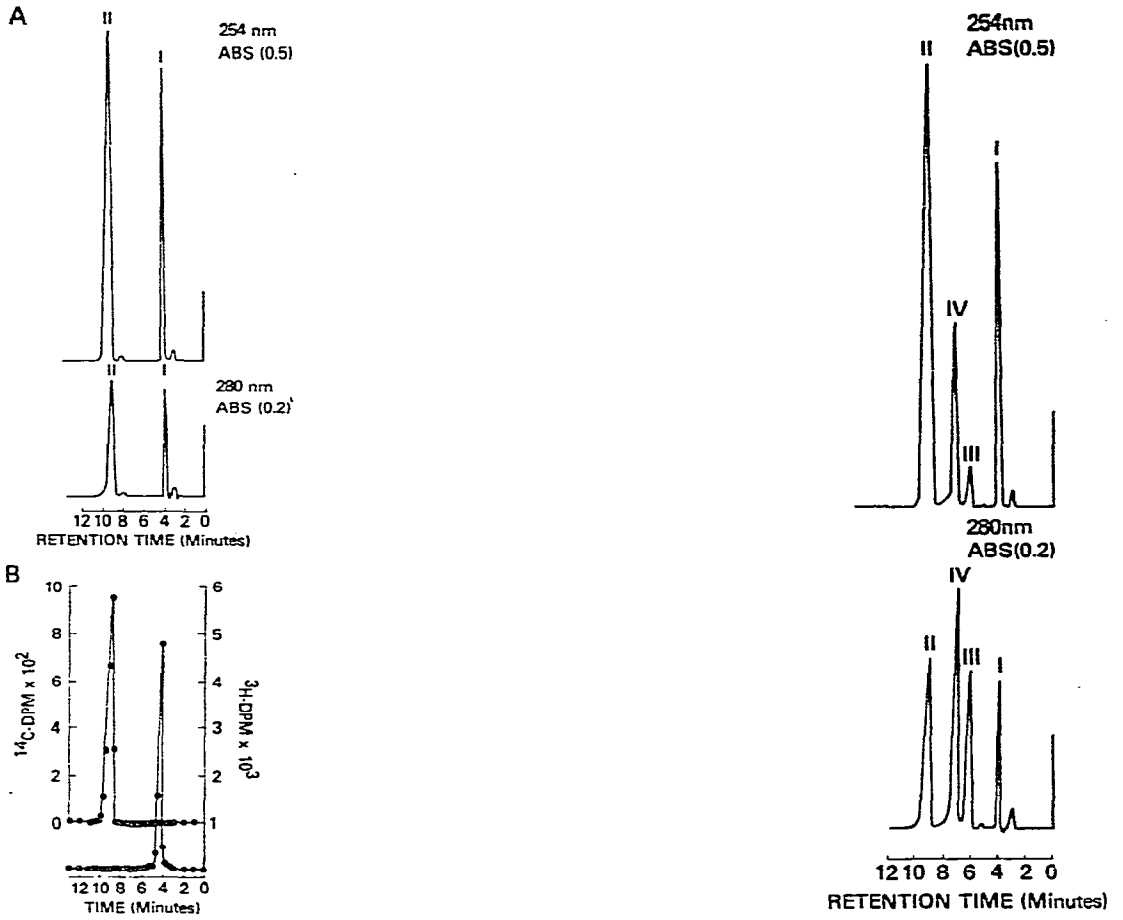


Fig. 2. The chromatograms for unlabeled acetaminophen and phenacetin and the radiolabeled internal standards. Absorbance (A) was measured at 254 nm and 280 nm after injection of an extracted sample containing 10 μg acetaminophen (I) and 25 μg phenacetin (II). The values in parentheses denote the sensitivities of the detector. The eluted (0.25 min) fractions of the radiolabeled internal standards, [^{3}H]acetaminophen and [^{14}C]phenacetin, are shown in (B).

Fig. 3. The chromatograms of acetaminophen, phenacetin, phenetidine and 2-hydroxyphenacetin. The absorbance was measured at 254 nm and 280 nm after injection of an extracted sample containing 10 μg of acetaminophen (I), 25 μg of phenacetin (II), 22.3 μg of phenetidine (III), 30 μg of 2-hydroxyphenacetin (IV) and 55 μg of N-hydroxyphenacetin. N-Hydroxyphenacetin was not eluted from the column.

multiplied by the absorbance unit used for the sample and divided by lowest absorbance unit used. The calibration curve was obtained by a regression curve of the peak heights of the samples divided by the radioactive counts collected for the internal standard against known amounts of the sample with a weighted regression program (Hewlett-Packard HP65 Standard-Pac 2, Stat 2-09A). A linear relationship was obtained for the calibration curve of acetaminophen (0.5–50 μg) on the first day of injection of the samples into the liquid chromatograph (Fig. 4). Upon reinjection of the same samples, linear relationships were also obtained for days 2, 3 and 5; the

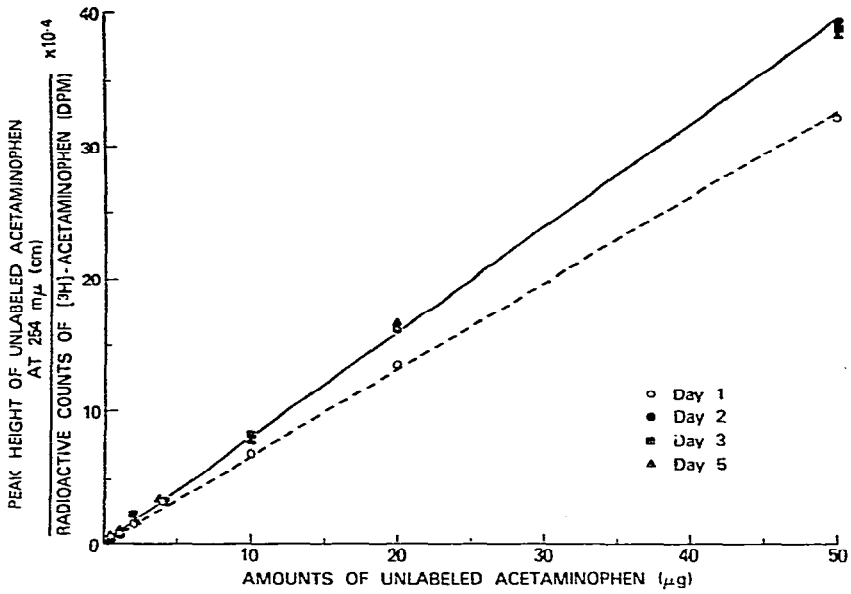
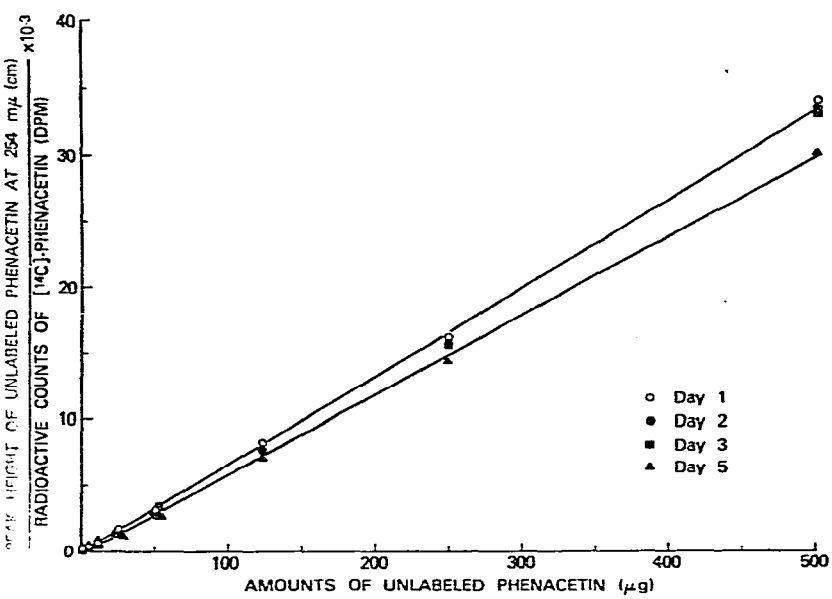


Fig. 4. Calibration curve for unlabeled acetaminophen at 254 nm with [^{3}H]acetaminophen as internal standard. Days 1, 2, 3 and 5 indicate the days of reinjection of the same set of standards.

slopes for days 2, 3 and 5 were virtually identical, but were different from that for day 1. A small intercept was obtained for all the calibration curves.

A linear relationship was obtained for the calibration curve of phenacetin (2.5–500 μg) on the first day of injection of the samples into the liquid chromatograph (Fig. 5). Upon reinjections of the same samples, linear relationships were also



g. 5. Calibration curve for unlabeled phenacetin at 254 nm with [^{14}C]phenacetin as internal ndard. Days 1, 2, 3 and 5 indicate the days of reinjection of the same set of standards.

TABLE I

THE SIMULTANEOUS QUANTITATION OF UNLABELED ACETAMINOPHEN AND PHENACETIN

	Known amounts of sample (μg)		*Assayed values from the calibration curve (μg)*		
	Acetaminophen	*Phenacetin*	*Day 1*	*Day 2*	*Day 5*
I	0.5		0.48 (3.7)*	0.49 (3.2)	0.49 (4.3)
		250	248 (5.4)	246 (3.7)	244 (4.9)
	($n = 10$)				
II	50		50.0 (3.7)	51.1 (4.9)	—
		25	26.2 (4.7)	24.9 (5.0)	—
	($n = 10$)				

* The number in parentheses is the coefficient of variation expressed in percent.

obtained for days 2, 3 and 5. The slopes for days 1, 2 and 3 were virtually identical, but were different from that for day 5. Again, a small intercept existed for all the calibration curves.

Ten samples containing both 0.5 μg acetaminophen and 250 μg phenacetin, and ten samples containing both 50 μg acetaminophen and 25 μg phenacetin, were extracted under similar conditions. The assayed values obtained from the calibration curves were in close agreement with their known values (Table I). The coefficients of variation of these samples were less than 5%.

Tracer amounts of radiolabeled acetaminophen and phenacetin

For the calibration curve, the radioactive counts of the samples divided by the peak height of the unlabeled internal standard were plotted against known

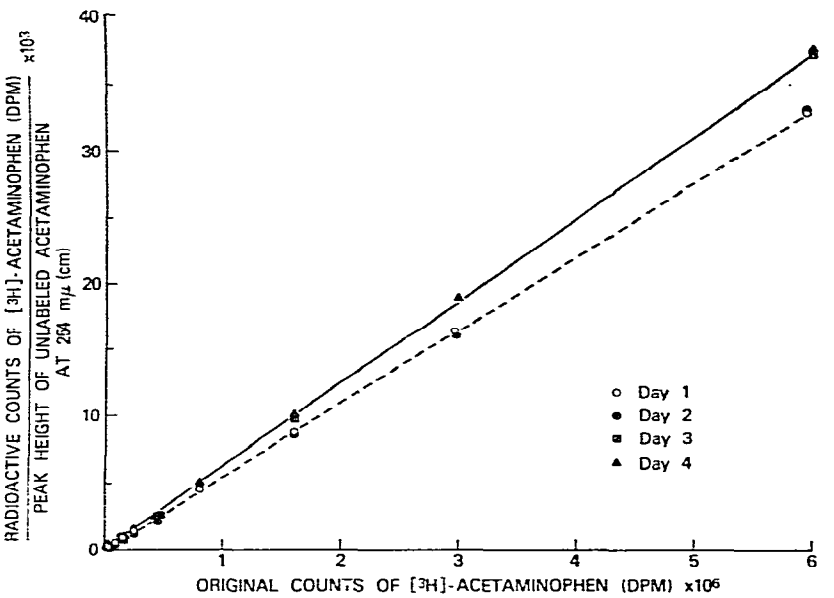


Fig. 6. Calibration curve for tracer amounts of radiolabeled [^{3}H]acetaminophen at 254 nm. Unlabeled acetaminophen was used as the internal standard. Days 1–4 indicate the days of reinjection of the same set of standards.

radioactive counts of the samples. A linear relationship was obtained for the calibration curve of [^{3}H]acetaminophen (0.036×10^6–12.15×10^6 dpm) for day 1 of injection of the samples, and upon reinjection of the same samples, for days 2, 3 and 4 (Fig. 6). The slope for day 1 was virtually identical to that for day 2, but was different from those of days 3 and 4. A very small intercept existed for all the calibration curves.

A linear relationship was obtained for the calibration curve of tracer amounts of [^{14}C]phenacetin (1.6×10^3–879.4×10^3 dpm) for day 1 of the injection of the samples, and upon reinjection of the same samples, for day 2, 3 and 4 (Fig. 7). Again, a very small intercept existed in all the calibration curves.

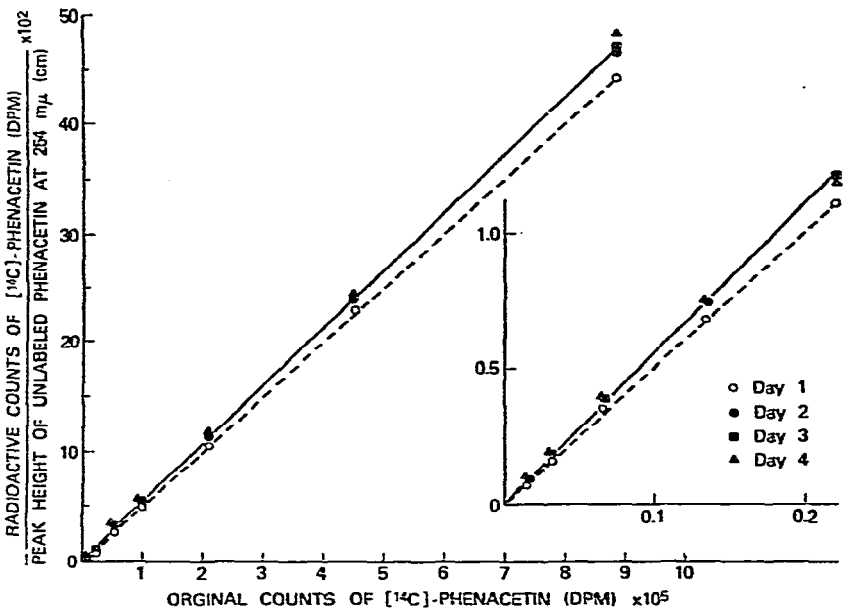


Fig. 7. Calibration curve for tracer amounts of radiolabeled [^{14}C]phenacetin at 254 nm. Unlabeled phenacetin was used as the internal standard. Days 1–4 indicate the days of reinjection of the same set of standards.

When ten samples containing both 0.18×10^6 dpm [^{3}H]acetaminophen and 866.8×10^3 dpm [^{14}C]phenacetin and ten samples containing both 12.15×10^6 dpm [^{3}H]acetaminophen and 6.45×10^3 dpm [^{14}C]phenacetin were assayed under similar conditions. The assayed values obtained from the calibration curves were in close agreement with the known values (Table II). The coefficients of variation of the samples were within 5%.

DISCUSSION

The use of a tracer amount of a radiolabeled drug of high specific activity as internal standardization for the same unlabeled drug appears to be a valid approach in HPLC. This approach is especially valuable when the response by an internal standard is undesirable. The radiolabeled drug is present in such small quantities in each sample that it is essentially invisible, and does not contribute to the peak height

TABLE II

THE SIMULTAENOUS QUANTITATION OF RADIOLABELED ACETAMINOPHEN AND PHENACETIN

	Known radioactivity of sample (dpm)		*Assayed values from the calibration curve (dpm)*		
	[^{3}H]-Acetaminophen	*[^{14}C]-Phenacetin*	*Day 1*	*Day 2*	*Day 4*
I	12.15×10^6		11.70×10^6 (3.4)*	11.10×10^6 (0.9)	11.83×10^6 (4.2)
		6.45×10^3	6.7×10^3 (4.6)	6.7×10^3 (4.7)	6.36×10^3 (0.8)
	($n = 10$)				
II	180×10^3		174×10^3 (3.4)	192×10^3 (4.5)	—
		867×10^3	857×10^3 (3.0)	837×10^3 (1.4)	—
	($n = 10$)				

* The number in parentheses is the coefficient of variation expressed in percent.

of the chromatogram. Sample collection for this invisible internal standard is possible in liquid chromatography, and is aided by the visual detection of the unlabeled drug on the recorder. Moreover, this invisible internal standard usually has the same solubility properties and stability as the unlabeled material. Our results indicated that the method is valid for the simultaneous quantitation of both unlabeled acetaminophen and phenacetin. The slope of the standard curves changed from day to day, but the reason for this is unknown.

The results also showed that the use of unlabeled compound as the internal standard for tracer amounts of the radiolabeled compound is valid. Indeed this method may be useful to quantitate tracer amounts of a radiolabeled compound in biological fluids.

In both instances, however, a balance between the amount of the internal standard and the amount of the compound in the assay has to be maintained. The amount of radiolabeled internal standard should be sufficiently low to remain invisible and yet furnish sufficient counts for the assay. Conversely, the amount of unlabeled drug for internal standardization should be high and at the maximum response of the UV detector of the liquid chromatograph such that any contribution from the radiolabeled drug to the peak height would hence become negligible. In both instances, the radiolabeled drug must not be selectively adsorbed onto the glassware on the column; recovery of the radioactive counts at the elution time interval must be complete and must correspond to the retention time of the sample.

Our method, unlike other published methods for acetaminophen and phenacetin[6–11], tested for possible interference by the metabolites. Buckpitt *et al.*[12] recently developed a method for the assay of radiolabeled acetaminophen, but this method was developed primarily for the assay of the radiolabeled thio-conjugates of acetaminophen, and did not include an extraction procedure or internal standards. In our method we utilized a simple extraction procedure without the use of special instrumentation. The method is sufficiently sensitive to detect low concentrations of acetaminophen (0.05 mg/l) and phenacetin (0.25 mg/l), and the calibration curves are linear over a wide concentration range (up to 500 mg/l for acetaminophen and up to 5 g/l for phenacetin); the calibration curve for radiolabeled acetaminophen was linear over a 165-fold change in radioactive counts, and that for radiolabeled phen-

acetin was linear over a 550-fold change in radioactive counts. The extraction procedure is very simple, and the analysis time by the liquid chromatograph is rapid (11 min).

ACKNOWLEDGEMENT

The authors would like to thank Dr. Lance R. Pohl of the Laboratory for the mass spectral analysis.

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