Journal of Chromatography, 208 (1981) 17–26
Elsevier Scientific Publishing Company, Amsterdam — Printed in The Netherlands

CHROM. 13,561

HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ANALYSIS OF THE MYCOTOXIN SECALONIC ACID D AND ITS APPLICATION TO BIOLOGICAL FLUIDS

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(Received October 23rd, 1980)

SUMMARY

Secalonic acid D (SAD) is an acutely toxic, teratogenic and possibly mutagenic fungal metabolite produced in corn by *Penicillium oxalicum*. Using ultraviolet absorbance at 340 nm as a means of detection, SAD was resolved as a sharp peak by reversed-phase high-performance liquid chromatography (HPLC) on a small particle (10 μm) μBondapak C_{18} column in 4 min by an acetonitrile–water–glacial acetic acid–tetrahydrofuran (5:3:0.5:0.5 for solvent system A and 4:3:0.5:0.5 for solvent system B) elution solvent system. The flow-rates for the two solvent systems (A and B) were 1.5 and 1.7 ml/min; column operating pressures of 1500 and 1800 p.s.i., respectively, resulted. The relationship between peak height or peak area and the quantity of SAD injected was linear over a range of 1–50 ng. Detection was very sensitive with lower limits of detection of 0.6 and 0.7 ng of SAD in solvent systems A and B, respectively. Retention times, peak heights and peak areas were highly reproducible in both solvent systems. Detection of SAD in spiked (0.2–10 μg/ml) urine and bile samples injected without cleanup onto the HPLC column and quantitative extraction of SAD by ethyl acetate from spiked, acidified plasma samples (0.1–5 μg/ml) followed by HPLC analysis were obtained. Solvent system B gave better resolution of SAD from the interfering substances in bile and urine than solvent system A. However, sensitivity was slightly greater in solvent system A.

INTRODUCTION

Secalonic acid D (SAD) is a toxic metabolite of *Pencillium oxalicum*, a fungus that commonly infects corn. Forty-four percent of pre-harvest corn in the midwestern U.S.A. has been reported to be infected with this fungus[1]. Acute toxicity[2], teratogenicity[3] and possible mutagenicity[4] of SAD in mice also have been reported. In view of these deleterious effects in experimental animals, a program designed to monitor the level of SAD in corn and food products made from corn is essential. At present, only

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0021-9673/81/0000–0000/$02.50

qualitative methods designed to identify the presence of SAD by thin-layer chromatography (TLC) are available[5,6]. This paper describes a high-performance liquid chromatographic (HPLC) method for the quantitation of SAD in bile, plasma and urine and a procedure for the extraction of SAD from urine.

EXPERIMENTAL

Toxin

SAD (Fig. 1), produced as described by Reddy *et al.*[2], was established to be 99% pure by melting point, nuclear magnetic resonance, mass spectroscopy and TLC. In addition, the purity of radiolabelled [^{14}C]SAD (>99%) was established by radiochromatography and HPLC. A 1-mg/ml stock solution of SAD was prepared in each solvent used in the investigation and appropriate dilutions were made by carefully diluting the stock solution with the respective solvent to give concentrations ranging from 0.2–10 μg/ml.

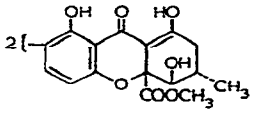

Fig. 1. Structure of one of the two identical halves of the secalonic acid D molecule, $C_{32}H_{30}O_{14}$. Molecular weight, 638.16.

Equipment

Both normal- and reversed-phase chromatography were performed using a Waters Assoc. HPLC system including a U6K septumless injector and a Model 440 fixed-wavelength absorbance detector (Water Assoc., Milford, MA, U.S.A.) and an Omniscribe dual-pen recorder equipped with an electronic integrator (Houston Instruments, Austin, TX, U.S.A.). Separations were performed either with a μPorasil (normal-phase) or μBondapak C_{18} (reversed-phase) column (particle size 10 μm, dimensions 30 cm × 3.9 mm I.D.) with flow-rates of 1–2 ml/min at a nominal pressure of 700–2000 p.s.i. SAD was detected at 340 nm, with absorbance detector sensitivities of 0.005–0.1 absorbance units full scale (AUFS). A pre-column (Whatman, Clifton, NJ, U.S.A.) was installed before the column for analysis of SAD from bile, plasma and urine samples.

Elution solvent systems

A comparison of the solvents and solvent combinations is listed in Table I. All solvents were distilled in glass (Burdick & Jackson Labs., Muskegon, MI, U.S.A.) unless otherwise specified. Each elution solvent system was degassed by stirring under vacuum after mixing appropriate ratios of individual solvents.

RESULTS AND DISCUSSION

Optimization of chromatography

Ultraviolet (UV)–visible absorbance spectra of SAD in acidic acetonitrile and alkaline aqueous (5% $NaHCO_3$) solutions, as given in Fig. 2, indicate that peak

TABLE I
PARAMETERS OF SECALONIC ACID D IN VARIOUS SOLVENT SYSTEMS ON A μBONDAPAK C_{18} COLUMN
$k' = (V_1 - V_0)/V_0$ where V_0 = void volume and V_1 = volume of the solvent required for peak elution.

Mobile phase	*Ratio (v/v)*	*Flow-rate (ml/min)*	*Retention time (t_R) (sec)*	*k'*
Acetonitrile	—	1.0	90	0.00
Acetonitrile–ethyl acetate	3:1	2.0	85	0.88
Acetonitrile–chloroform	9:1	1.5	87	0.45
Acetonitrile–water	1:1	1.0	98	0.09
Acetonitrile–water–glacial acetic acid	100:4:5	1.5	133	1.22
Acetonitrile–water–glacial acetic acid	5:1:0.5	1.5	160	1.67
Acetonitrile–water–glacial acetic acid	7:3:0.5	1.5	210	2.50
Acetonitrile–water–glacial acetic acid	5:3:0.5	1.5	337	4.61
Acetonitrile–water–glacial acetic acid–tetrahydrofuran	5:3:0.5:0.5	1.5	239	3.00
Acetonitrile–water–glacial acetic acid–tetrahydrofuran	4:3:0.5:0.5	1.7	240	3.53

maxima of SAD are at 339 and 360 nm, respectively. In the alkaline solution, a rapid drop in absorbance of SAD (to 10% of the original) occurred when measured at 360 nm. A bathochromic shift in the peak maximum (to 390 nm) occurred which was almost complete by 48 h (Fig. 3). Under acid conditions, however, neither a drop in absorbance nor a shift in the peak maximum occurred. These data suggested the need to maintain the solvent system in an acid condition during the preparation of samples

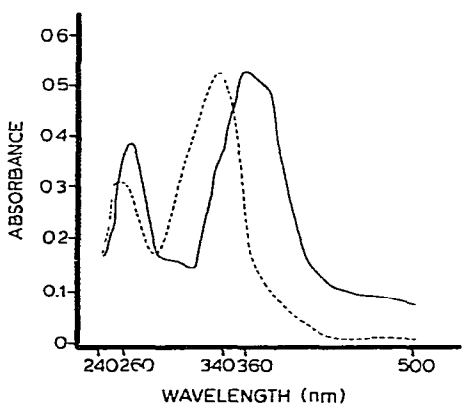

Fig. 2. UV–visible absorbance spectra of secalonic acid D (10 ng/ml) in acidic acetonitrile (——, pH 2.0) or aqueous 5% (w/v) sodium bicarbonate (----, pH 8.0).

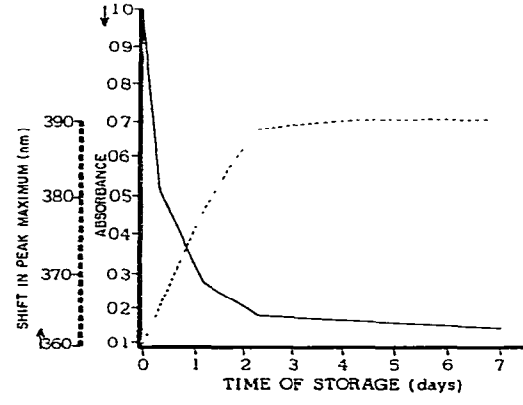

Fig. 3. Instability of secalonic acid D in alkaline (pH 12.0) sodium hydroxide. The solid line indicates the drop in absorbance at 360 nm and the broken line indicates the shift in the peak maximum from 360 to 390 nm, upon storage.

for HPLC analysis. Furthermore, acidic conditions (6% oxalic acid slurry over the silica gel plate) were essential for the TLC separation of SAD[5,6].

The solvent system that gave the best TLC separation (without tailing) of SAD[5] (toluene–ethyl acetate–90% formic acid, 6:3:1) also eluted SAD as a sharp peak on a μPorasil column (not shown). The pH of this solvent system, however, was below 2, a condition that would damage the column on repeated use. Also the retention time (t_R) and the capacity ratio (k') for the eluted peak were unacceptable. Reduction in the amount of formic acid to bring the pH of the solvent to a level (above 2) condusive for column longevity resulted in reduced sensitivity as well as broadening and tailing of the peak. The other solvent systems used with the μPorasil column also were unsatisfactory.

The retention time and the capacity ratio for SAD in several solvent systems used in conjunction with a reversed-phase column are listed in Table I. In general, all of the solvent systems that did not contain glacial acetic acid yielded unacceptable t_R and k' values. The detection of SAD in these systems also was less sensitive. Among those solvent systems containing acetic acid, the best t_R and k' values were obtained with acetonitrile–water–glacial acetic acid (5:3:0.5). However, the SAD peak was broad and sensitivity was low. Although the addition of tetrahydrofuran to this solvent system (acetonitrile–water–glacial acetic acid–tetrahydrofuran, 5:3:0.5:0.5; referred to as solvent system A) compromised the k' value to some extent, it remained within acceptable limits. The peak for authentic SAD was sharp (Fig. 4) and the sensitivity good. Decreasing the ratio of acetonitrile (to 4:3:0.5:0.5; solvent system B) reduced sensitivity (Table I), but resulted in better resolution of SAD from interfering peaks in urine and plasma extracts (Figs. 5 and 6). Solvent systems A and B were the solvent systems used in the rest of the investigation. Flow-rates of 1.5 and 1.7 ml/min, respectively, were used.

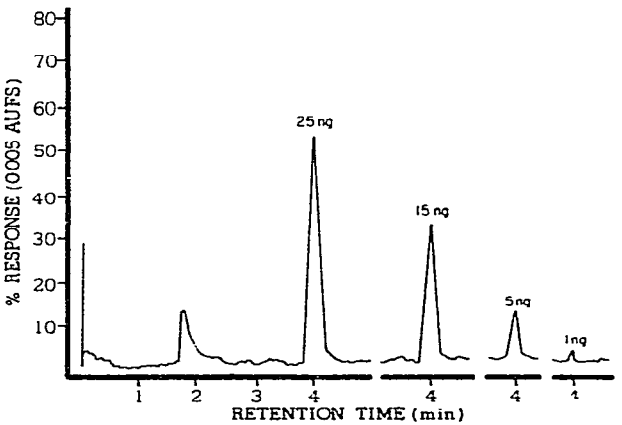

Fig. 4. HPLC resolution of secalonic acid D on a μBondapak C_{18} column with elution solvent system A (acetonitrile–water–glacial acetic acid–tetrahydrofuran, 5:3:0.5:0.5; flow-rate 1.5 ml/min), detection at 340 nm.

Detection

The UV–visible spectra of SAD in solvent systems A and B were identical to the UV–visible spectrum observed in acidic acetonitrile (Fig. 2). The detection wave-

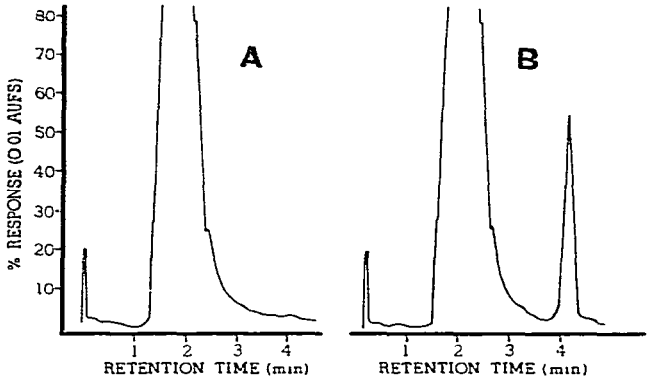

Fig. 5. Chromatograms of urine (5 μl) without (A) or with (B) 50 ng of secalonic acid D (10 μg/ml) in solvent system A (see text for column and conditions).

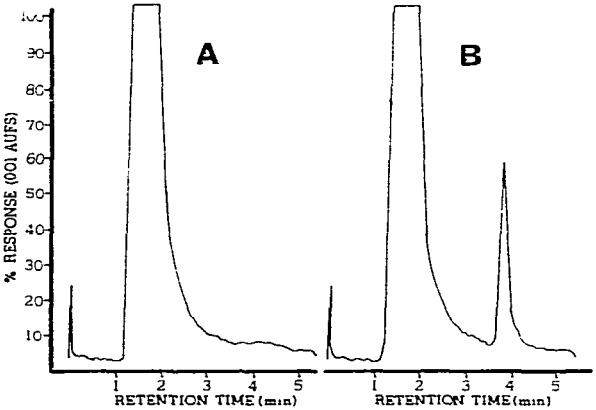

Fig. 6. Chromatograms of bile (5 μl) without (A) or with (B) 50 ng of secalonic acid D (10 μg/ml) in solvent system B (see text for column and conditions).

length of 340 nm, therefore, was chosen because it was the filter closest to the absorption maximum of 339 nm for SAD. The molar extinction coefficient, ε, at 340 nm for SAD closely approximated the $\varepsilon_{max.}$ for SAD in either mobile phase (solvent system A or B) or acetonitrile (Table II).

TABLE II
ABSORBANCE CHARACTERISTICS OF SECALONIC ACID D IN ACETONITRILE OR TWO HPLC ELUTION SOLVENT SYSTEMS
System A: acetonitrile–water–glacial acetic acid–tetrahydrofuran (5:3:0.5:0.5); flow-rate: 1.5 ml/min. System B: acetonitrile–water–glacial acetic acid–tetrahydrofuran (4:3:0.5:0.5); flow-rate: 1.7 ml/min.

Characteristic	*Acetonitrile*	*Solvent system A or B*
$\lambda_{max.}$ (nm)	339	339
$\varepsilon_{max.}$, 339 nm	33,000	32,450
ε, 340 nm	32,700	32,050

Retention time

Retention times were highly reproducible with both solvent systems (A or B) using the μBondapak C_{18} column (Table III). Thirty-two and 30 injections over a period of 3 days gave mean retention times of 239 and 240 sec, respectively, for the two solvent systems with coefficients of variation [(σ/mean) · 100] of 1.29 and 0.68%, respectively.

TABLE III
REPRODUCIBILITY OF RETENTION TIME FOR SECALONIC ACID D BY HPLC
System A: acetonitrile–water–glacial acetic acid–tetrahydrofuran (5:3:0.5:0.5); flow-rate: 1.5 ml/min.
System B: acetonitrile–water–glacial acetic acid–tetrahydrofuran (4:3:0.5:0.5); flow-rate: 1.7 ml/min.

Statistic	*Elution solvent system*	
	A	B
Injection (*N*)*	32	30
Flow-rate (ml/min)	1.5	1.8
Retention time (sec)		
Range	233–243	237–242
Mean	239	240
Mean retention time (min)	4.0	4.0
Standard deviation (sec)	3.11	1.63
Coefficient of variation (%)**	1.29	0.68

* Successive injections of secalonic acid D (0.005–0.1 μg) over 3 days.

** $\frac{\text{Standard deviation}}{\text{Mean}} \times 100.$

Linearity

The relationship between peak height (Fig. 7) and peak area and the amount of SAD injected, regardless of the solvent system (A or B), was linear over a range of 1–50 ng. The correlation for both parameters in either solvent system exceeded 0.999.

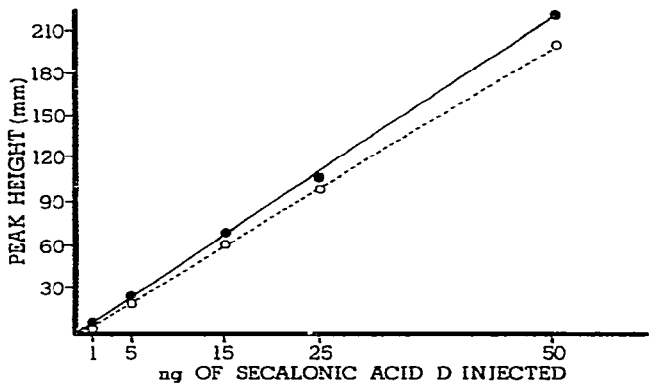

Fig. 7. Linearity, peak height *vs.* quantity of secalonic acid D (1–50 ng) at 340 nm in: (●) solvent system A (see Fig. 4 for composition and flow-rate) or (○) solvent system B (acetonitrile–water–glacial acetic acid–tetrahydrofuran, 4:3:0.5:0.5; flow-rate, 1.7 ml/min).

Precision and sensitivity

Precision was evaluated by injecting ten 5-μl aliquots of the SAD standard containing 25 ng of SAD in both solvent systems. Reproducibility of peak height and peak area was good, with coefficients of variation of 1.0–1.2% for peak height and 1.6–1.7% for peak area, representing the combined errors of HPLC resolution, injection and detection (Table IV).

TABLE IV
PEAK HEIGHT AND PEAK AREA REPRODUCIBILITY OF SECALINIC ACID D SEPARATION BY HPLC
System A: acetonitrile–water–glacial acetic acid–tetrahydrofuran (5:3:0.5:0.5); flow-rate: 1.5 ml/min.
System B: acetonitrile–water–glacial acetic acid–tetrahydrofuran (4:3:0.5:0.5); flow-rate: 1.7 ml/min.

Statistic	*Elution solvent system*	
	A	*B*
Injections (*N*)*	10	10
Peak height (mm)		
Range	107–110	96–110
Mean	108	98
Standard deviation (mm)	1.1	1.2
Coefficient of variation (%)**	1.0	1.2
Sensitivity (mm/ng)***	4.3	3.9
Detection limit (ng) §	0.6	0.7
Peak area (mm^2)		
Range	503–522	458–475
Mean	513	466
Standard deviation (mm^2)	8.2	8.1
Coefficient of variation (%)**	1.6	1.7
Sensitivity (mm^2/ng)***	20.5	18.6

* Successive injections of 25 ng secalonic acid D in 5 μl of an elution solvent system.

** $\frac{\text{Standard deviation}}{\text{mean}} \times 100.$

*** Calculated to maximum sensitivity, 0.005 a.u.f.s.

§ Detection limit is defined as the quantity of secalonic acid D which gave a peak height 2.5 times the background.

Mean sensitivity of SAD detection was 4.3 and 3.9 mm/ng of peak height and 20.5 and 18.6 mm^2/ng of peak area for solvent systems A and B, respectively (Table IV). Detection limit, defined as the quantity of SAD which would give a peak height 2.5 times the background, was calculated to be 0.6 ng in solvent system A and 0.7 ng in solvent system B (Table IV).

BIOLOGICAL APPLICATIONS

Analysis of SAD in rat urine and bile

Rat urine samples were analyzed before and after the addition of known quantities of SAD. Chromatograms of urine (5 ml) with and without 50 ng SAD using

solvent systems A and B as the eluting systems indicated that adequate separation of SAD from the polar components was obtained by using solvent system A (Fig. 5). Chromatograms of 5-μl aliquots of bile, however, showed inadequate resolution of SAD from the polar components in solvent system A (not shown). Resolution obtained with solvent system B, however, gave good separation of SAD from the polar compounds in bile (Fig. 6). A pre-column just before the μBondapak column increased the retention time of SAD by only 1 sec but allowed direct injection of urine and bile without sample cleanup. Repeated injections of these samples over a period of several days resulted in only a small increase (100 p.s.i.) in column pressure. This problem could be eliminated by regular washing of the column with 25 ml of degassed glass distilled water followed by 25 ml of acetonitrile at the end of the day's run.

Detection of 1–50 ng of SAD (0.2–10 μg/ml) in 5-μl aliquots of urine (solvent system A) and bile (solvent system B) was quantitative and linear. The elimination of the extraction step not only prevented loss of SAD that occurred during sample preparation, but may also allow for the detection and quantitation of metabolite(s) when radiolabelled SAD is administered to experimental animals.

Extraction of SAD from plasma

Rat plasma (0.1 ml) containing 10–500 ng of SAD was acidified with an equal volume of 1 *N* HCl. The mixture was extracted twice with 1 ml of ethyl acetate by mixing on a vortex for 2 min each time. The ethyl acetate extracts were pooled and evaporated under nitrogen. The residue was taken up in 50 μl of the mobile phase and 5-μl aliquots (containing 1–50 ng) were analyzed by HPLC using solvent system A.

Typical chromatograms of a plasma extract with or without 50 ng of SAD in solvent system A are shown in Fig. 8. Good separation of SAD from the ethyl acetate extractable components of the plasma was obtained. The recovery of SAD from plasma was 96.3 to 107.5% over a range of 0.1–5 ng of SAD/ml (Table V).

To determine the purity of [^{14}C]SAD, 5 ng of [^{14}C]SAD were injected and eluted with solvent system A. Fractions collected at 10-sec intervals were added to 10 ml of PCS (Amersham, Arlington Heights, IL, U.S.A.) for liquid scintillation spectrometry. All radioactivity eluted as a single peak (Fig. 9, A and B) corresponding to

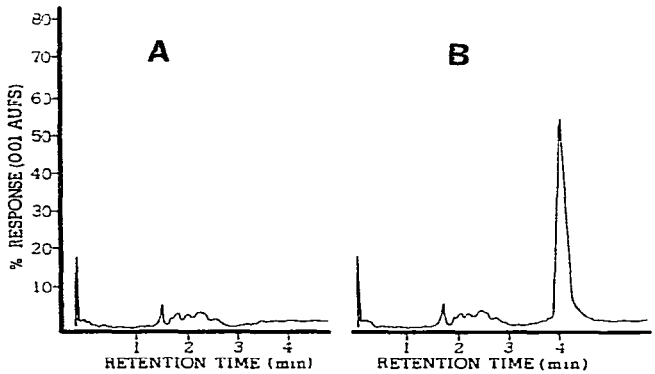

Fig. 8. Chromatograms of plasma extract without (A) or with (B) 50 ng of secalonic acid D (5 μg/ml of plasma) in solvent system A (see text for column and conditions).

TABLE V
RECOVERY OF SECALONIC ACID D FROM RAT PLASMA

Secalonic acid D (μg/ml)	*Recovery (%) $\bar{x} \pm$ S.E.*
0.1	103.4 ± 4.3
0.2	99.1 ± 3.9
0.5	107.5 ± 6.8
1.0	100.3 ± 5.0
2.0	98.0 ± 3.7
5.0	96.3 ± 5.2

the retention time of SAD (4 min; fraction 24) plus a 25-sec lag time between UV detection and collection of the fraction. A 100-μg amount of [^{14}C]SAD was added to 100 μl of plasma. The plasma then was extracted and analyzed by HPLC as described above. Fractions were collected and the radioactivity in each fraction determined. The elution profile of radioactivity from [^{14}C]SAD and its corresponding HPLC profile (Fig. 9, C and D) indicated that all of the radioactivity was extracted as free SAD.

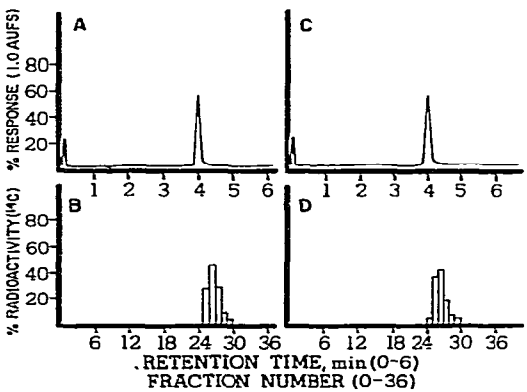

Fig. 9. Chromatograms of 5 μg of [^{14}C]secalonic acid D (A); or 5 μl of plasma extract containing [^{14}C]secalonic acid D (C) and their respective radioactivity profiles (B and D). Fraction collected at 10-sec intervals beginning at the time of injection. See text for explanation.

The methods described for the quantitative determination of SAD from bile, plasma and urine are rapid, simple and extremely sensitive. The methods allow for direct HPLC analysis of SAD in bile and urine and for a simple one-step extraction of SAD from plasma prior to HPLC analysis. These characteristics make this method suited for field monitoring of SAD plasma levels in farm animals and humans and/or for pharmacokinetic studies.

ACKNOWLEDGEMENTS

This research was supported by USPHS Grants ES01351 and ES02191. C.S.R. was supported by Training Grant ES07045.

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