liquid diffusion layers were employed in the theoretical analysis (1, 3). Rather, this work demonstrates that such diffusion layers are in reality convenient "fictions," which need not be invoked with certain experimental designs. Certainly, the concept of aqueous diffusion layers has been instrumental in the understanding of membrane transport processes, but too much credence has been given to their actual existence.

Biological analogies to these polymeric tube transport studies are the isolated perfusion and *in situ* techniques for intestinal absorption. An isolated intestinal segment suspended as nearly straight as possible would serve as a means for determining the luminal convective diffusion contribution to intestinal transport. For an *in situ* intestinal system, definite bends in the intestine would induce secondary flows or turbulence, which would require that absorption data be carefully analyzed for the demarcation between convective diffusion control and membrane control. With the ability to determine accurately the luminal contribution to overall intestinal transport, absorption models can be established on a firmer physical basis. Without the need for semiempirical approaches to obtain diffusion layer or stagnant film permeability coefficients, the elucidation of membrane transport characteristics should be facilitated.

Furthermore, the tube configuration is of value pharmaceutically for obtaining the intrinsic dissolution rates of solids (9). Solid tubes, obtained by various methods, can be infused with dissolution media and the efflux concentrations can be measured as a function of flow rate. From such information, the presence or absence of surface or other kinetic barriers can be determined by comparison of efflux concentrations with the theoretical predictions for pure convective diffusion control.

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ACKNOWLEDGMENTS AND ADDRESSES

Received February 9, 1976, from the *School of Pharmacy, University of Connecticut, Storrs, CT 06268,* and the ‡*Pharmacy Research Unit, The Upjohn Company, Kalamazoo, MI 49001.*

Accepted for publication May 5, 1976.

Support for this study in the form of a Summer Visiting Professorship from The Upjohn Co. is gratefully acknowledged by D. R. Flanagan.

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Quantitative Determination of Phenacetin and Its Metabolite Acetaminophen by GLC–Chemical Ionization Mass Spectrometry

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Abstract □ A quantitative GLC–mass spectrometric procedure was developed for the determination of phenacetin and its *O*-desethyl metabolite, acetaminophen, in human plasma. The assay utilizes selective ion detection to monitor, in a GLC effluent, the MH^+ molecular ions of both phenacetin and the methyl derivative of acetaminophen, *p*-acetanisidine, generated by isobutane chemical ionization. Deuterated analogs of phenacetin and acetaminophen, phenacetin-d_3 and acetaminophen-d_3, respectively, are added to the plasma before extraction to serve as internal standards. To determine phenacetin and unconjugated acetaminophen, 1.0 ml of plasma is extracted with 5 ml of benzene–dichloroethane (7:3). The extraction solvent is removed, and the residue is methylated with diazomethane. The solution is again evaporated to dryness, and the residue is reconstituted in ethyl acetate. A portion of this solution is then analyzed by GLC–mass spectrometry, with the mass spectrometer set to monitor *m/e* 166 (*p*-acetanisidine), 169 (*p*-acetanisidine-d_3), 180 (phenacetin), and 183 (phenacetin-d_3). To determine total acetaminophen, 0.1 ml of plasma is treated with a mixture of β-glucuronidase and sulfatase, extracted with ethyl acetate, methylated, and analyzed by GLC–mass spectrometry. The procedure has a sensitivity limit of 1 ng of phenacetin/ml and 0.1 μg of acetaminophen/ml. The curves relating the amount of phenacetin and acetaminophen added *versus* the amount of phenacetin and acetaminophen found for 12 known phenacetin concentrations over the 9.9–246.6-ng/ml range and for 16 known acetaminophen concentrations over the 0.52–13.10-μg/ml range are straight lines with intercepts of nearly zero and with slopes of unity. Analyses of six separate plasma samples, each containing 25 ng of phenacetin/ml and 1.31 μg of acetaminophen/ml, had a precision of ±1 ng/ml for phenacetin and ±0.08 μg/ml for acetaminophen.

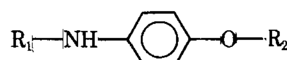
Keyphrases □ Phenacetin—GLC–mass spectrometric analysis, human plasma □ Acetaminophen—GLC–mass spectrometric analysis, human plasma □ GLC–mass spectrometry—analyses, phenacetin and acetaminophen, human plasma □ Analgesics—phenacetin and acetaminophen, GLC–mass spectrometric analysis, human plasma

Various nonspecific spectrophotometric (1–5) procedures or time-consuming liquid (6, 7), paper (8, 9), or thin-layer (10–12) chromatographic procedures have been developed to measure the analgesic phenacetin (I) and its analgesic biotransformation product, acetaminophen (II), in human plasma. The published GLC procedures for these compounds have a detection limit of 50 ng/ml (13–15). While this sensitivity is suitable for the measurement of

Figure 1—*Isobutane mass spectra of phenacetin,* p-*acetanisidine, acetaminophen, and their deuterated analogs.*

acetaminophen, it permits the determination of phenacetin concentrations in normal subjects for only a few hours after oral administration of a therapeutic phenacetin dose and is insufficient for phenacetin determinations in many subjects with induced drug-metabolizing capabilities (16, 17).

This report describes a GLC–mass spectrometric assay that requires 1.1 ml of plasma and is capable of determining 1 ng of phenacetin/ml and 0.1 μg of unconjugated and conjugated acetaminophen/ml. The method utilizes chemical ionization (18) GLC–mass spectrometry, with isobutane functioning both as a reagent and GLC carrier gas. To obtain sufficient sensitivity, selective ion detection is used to monitor the MH^+ ion of both phenacetin and the methyl derivative of acetaminophen, *p*-acetanisidine (III). Deuterated analogs of phenacetin and acetaminophen, phenacetin-d_3 (IV) and acetaminophen-d_3 (V), respectively, are added to the plasma as internal standards.



I: $R_1 = CH_3CO$, $R_2 = CH_2CH_3$
II: $R_1 = CH_3CO$, $R_2 = H$
III: $R_1 = CH_3CO$, $R_2 = CH_3$
IV: $R_1 = CH_3CO$, $R_2 = CH_2CD_3$
V: $R_1 = CD_3CO$, $R_2 = H$
VI: $R_1 = CD_3CO$, $R_2 = CH_3$

EXPERIMENTAL

Apparatus—A quadrupole mass filter[1] and data system[2] were used in conjunction with a gas chromatograph[3]. The GLC column, 1.21 m (4 ft) × 2 mm i.d., was silanized, packed with 3% OV-17 on 100–120-mesh Gas Chrom Q[4], and conditioned at 325° with no flow for 3 hr and then at 275° overnight with a 20-ml/min helium flow. The chromatographic conditions were: column oven, 180–195°; injection port, 300°; isobutane[5] pressure, 0.45–1.36 kg (1–3 lb)/in.^{2}; and interface oven and glass-lined source reentrant tube, 250°. Typical ion source settings were: ion volume, +8 v; ion repeller, +2 v; lens, −30 v; and filament, −110 v with the electron beam total emission regulated at 800 μamp. Column temperature and isobutane pressure were adjusted to provide an ion source pressure of 0.4 torr. The source was operated without external heating (temperature approximately 50°).

Materials—Nanograde quality benzene[6], methanol[6], and ethyl acetate[6] and spectrograde quality 1,2-dichloroethane[7] were used without further purification. Before each assay, ethereal diazomethane (50 ml) was prepared according to Arnt's (19) decanting procedure and dried over anhydrous sodium sulfate.

Tubes (15 ml)[8] equipped with screw caps[9] were used for the plasma extractions, and glass-stoppered centrifuge tubes (5 ml)[10] were used for the final solvent evaporation.

Phenacetin-d_3—A mixture of 4-hydroxyacetanilide (5.0 g, 32 mmoles),

[1] Finnigan model 1015D.
[2] Finnigan model 6000.
[3] Finnigan model 9500.
[4] Applied Science Laboratories.
[5] Matheson, instrument purity.
[6] Mallinckrodt Chemicals.
[7] Fisher Scientific.
[8] Pyrex 9825.
[9] Lined with Teflon (du Pont).
[10] Pyrex 8084.

potassium carbonate (2.8 g, 20 mmoles), ethyl iodide-d_3 (3 ml, 35 mmoles)[11], and acetone (20 ml) was refluxed for 20 hr (20). The solution was cooled, transferred to a separator, and combined with water (20 ml), chloroform (75 ml), and 10% potassium hydroxide (5 ml). The chloroform extract was washed twice with 10% potassium hydroxide (10 ml), the chloroform was removed, and the resulting gum was crystallized from methanol–water to yield 3.85 g (21 mmoles, 66%) of phenacetin-d_3, mp (uncorrected) 131–132° [lit. (21) mp (phenacetin) 134–135°].

The UV (methanol) and IR (potassium bromide) spectra were identical to those of authentic phenacetin, while the PMR[12] spectrum (deuterochloroform) differed from that of phenacetin only by the lack of the triplet at 1.4 ppm. A precise mass determination[13] of the *m/e* 182 molecular ion in the electron ionization mass spectrum gave 182.1137 ($C_{10}H_{10}D_3N_1O_2$ = 182.1135). A selective ion detection analysis of this compound using the developed GLC–mass spectrometric procedure showed the presence of less than 0.5% phenacetin-d_0.

p-*Hydroxyacetanilide*-d_3—*p*-Hydroxyaniline (535 mg, 5 mmoles), 12 *M* HCl (71 µl), deuterium oxide (8.43 ml)[11], 0.2 *M* sodium acetate (5 ml), acetic anhydride-d_6 (510 mg, 468 µl, 5 mmoles)[11], and ether (10 ml) were combined in a 25-ml separator. The mixture was gently shaken every 5 min for 1 hr (8). Then the aqueous layer was removed, and the ether was extracted with 0.5 *M* potassium hydroxide (3 × 5 ml) and discarded. The combined alkaline extracts were then acidified to pH 1 and extracted with ethyl acetate (3 × 10 ml). The residue remaining after removal of the ethyl acetate was recrystallized from water to yield 387 mg (2.56 mmoles, 51%) of the title compound, mp (uncorrected) 166–168° [lit. (22) mp (acetaminophen) 169–170°].

The UV (ethanol) and IR (potassium bromide) spectra were identical to those of acetaminophen, while the PMR spectrum (deuterochloroform) differed from that of acetaminophen only by the lack of the three-proton singlet at 2.07 ppm. A precise mass determination[13] of the *m/e* 154 molecular ion in the electron ionization mass spectrum gave 154.0841 ($C_8H_6D_3NO_2$ = 154.0821). A selective ion detection analysis of this compound using the described GLC–mass spectrometric procedure showed the presence of less than 0.5% *p*-hydroxyacetanilide-d_0.

Mass Spectrometry—The isobutane chemical ionization mass spectra of phenacetin, phenacetin-d_3, acetaminophen, acetaminophen-d_3, acetanisidine, and acetanisidine-d_3 can be seen in Fig. 1. The spectra are quite simple, consisting principally of MH^+ molecular ions, and were obtained at 0.4 torr by the separate injections of 10 µg of each compound into the GLC–mass spectrometer with the electron multiplier run at low gain to deemphasize background ions resulting from column bleed.

If both phenacetin and acetaminophen were to be determined, the mass spectrometer was set to monitor the MH^+ molecular ions at *m/e* 166 (acetanisidine), 169 (acetanisidine-d_3), 180 (phenacetin), and 183 (phenacetin-d_3). To measure only acetaminophen, the instrument was set to monitor *m/e* 166 and 169.

Assay Procedure—*Phenacetin and Unconjugated Acetaminophen (Procedure A)*—To 1.0 ml of plasma in a 15-ml tube was added 1.0 ml of internal standard solution containing 25–300 ng of phenacetin-d_3/ml and 2.5 µg of acetaminophen-d_3/ml. The resulting solution was allowed to stand for 2 hr. Then 5 ml of benzene–1,2-dichloroethane (7:3) was added, and the mixture was shaken[14] for 20 min. After centrifugation[15] for 20 min at 150×*g*, 4.0 ml of the organic layer was transferred to a 5-ml centrifuge tube. The extraction solvent was then removed at 30° under a gentle stream of nitrogen[16].

The resulting residue was dissolved in 0.5 ml of methanol and treated with 0.5 ml of a diazomethane–ether solution. This mixture was vortexed[17] and allowed to stand for 2 hr. The solvents were removed under a stream of nitrogen, and the residue was reconstituted in 30 µl of ethyl acetate. Depending on the expected phenacetin and acetaminophen concentrations, between 0.5 and 5 µl was injected into the gas–liquid chromatograph.

Thirty seconds after injection, the electron multiplier was turned on and the GLC divert valve was turned off, allowing the GLC effluent to enter the ion source. Forty-five seconds after injection, the ion source supplies were turned on; 15 sec later, computer-controlled scanning and data collection were initiated. Approximate retention times were: *p*-acetanisidine, 1.8 min; and phenacetin, 2.3 min. Three minutes after injection, the GLC divert valve was turned on, the ion source supplies were turned off, and the computer data collection was halted.

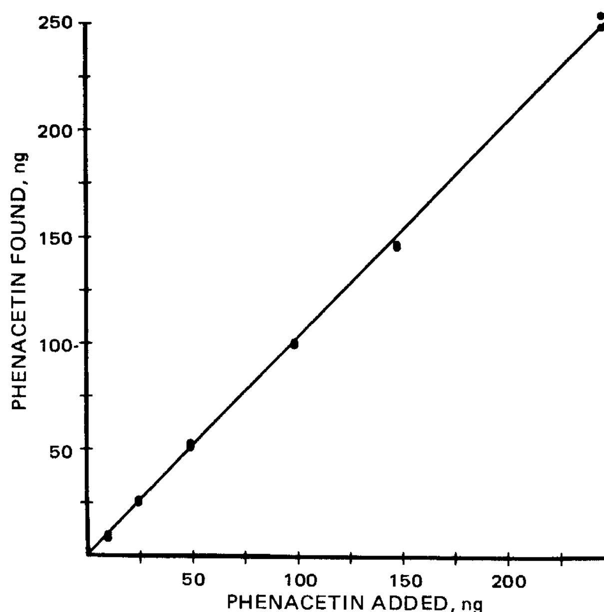


Figure 2—*Curve relating amount of phenacetin added to 1 ml of human control plasma versus amount of phenacetin found using Procedure A for two separate analyses at each of six different concentrations. The concentration of the phenacetin-*d_3 *internal standard was 50.5 ng/ml.*

To determine the phenacetin concentration, the area of the *m/e* 180 (phenacetin) peak was divided by that of the *m/e* 183 (phenacetin-d_3) peak and the ratio was multiplied by that of the phenacetin-d_3 concentration. A similar procedure was followed for acetaminophen, except peak heights instead of peak area were used.

Total Acetaminophen (Procedure B)—Plasma (0.1 ml), a β-glucuronidase–sulfatase mixture[18] (0.1 ml), 0.2 *M* sodium acetate pH 5.0 buffer (1 ml), distilled water (0.6 ml), and 0.5 µg of acetaminophen-d_3/ml (1 ml) were incubated with shaking[19] for 16 hr at 37°. The mixture was extracted for 20 min with 8 ml of ethyl acetate and centrifuged at 150×*g* for 15 min. A portion (5 ml) of the organic phase was removed and evaporated to dryness under nitrogen.

The residue was methylated and reconstituted in 30 µl of ethyl acetate, and 1 µl of the solution was injected into the GLC–mass spectrometer. The GLC and mass spectrometric parameters were identical to those used in Procedure A, except the mass spectrometer was set to monitor only *m/e* 166 and 169.

Samples—Plasma standards were prepared by the addition of known amounts of phenacetin and acetaminophen to human control plasma[20].

A 72-kg white male received an oral dose of phenacetin (900 mg)[21] as a fine powder followed by water (300 ml). A heparinized whole blood sample was drawn just before dosing and then every hour for 8 hr. The blood was centrifuged for 20 min at 150×*g*, and the plasma was removed for analysis.

RESULTS AND DISCUSSION

To measure phenacetin and unconjugated acetaminophen, 1 ml of plasma was extracted with benzene–1,2-dichloroethane (7:3). Recoveries for this extraction procedure (Procedure A), determined from a comparison of the peak areas of the internal standards obtained from six separate analyses with the areas from the injection of known amounts of diazomethane-treated phenacetin-d_3 and acetaminophen-d_3, were 95 ± 8% for phenacetin and 0.91 ± 0.12% for acetaminophen. This 104-fold greater extraction of phenacetin permitted both compounds to be determined from a single injection in spite of the commonly observed 100–1000-fold greater plasma acetaminophen concentration.

To determine total acetaminophen, 0.1 ml of plasma was treated with a β-glucuronidase–sulfatase mixture[18] and the acetaminophen was ex-

[11] Stohler Isotopes.
[12] Varian HA-100.
[13] CEC 21-110.
[14] International bottle shaker, International Equipment Corp.
[15] International centrifuge model K.
[16] N-Evap, Organomation Associates.
[17] Vortex Genie, Scientific Industries.
[18] Glusulase, Endo Laboratories Inc.
[19] Lab-Line, No. 3581.
[20] Hyland Laboratories.
[21] Eli Lilly, Indianapolis, Ind.

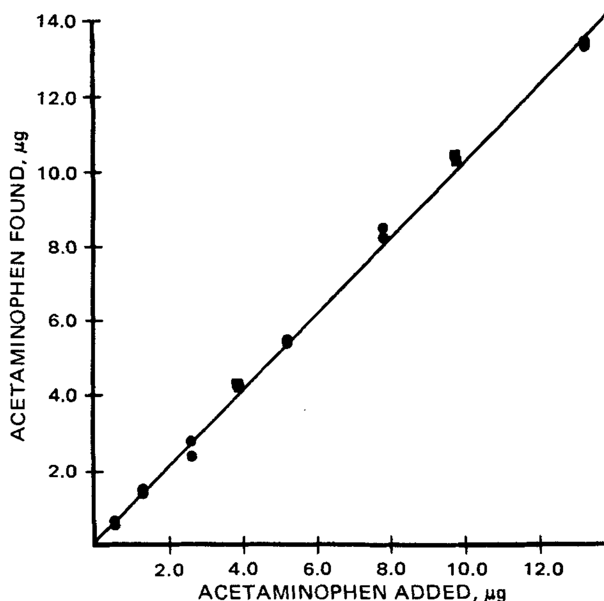


Figure 3—*Curve relating amount of acetaminophen added to 1 ml of human control plasma* versus *amount of acetaminophen found for two separate analyses at each of eight different concentrations. Circles are analyses using Procedure A with 2.52 µg of acetaminophen-*d_3*/ml used as the internal standard. Squares are determinations using Procedure B with 5.00 µg of acetaminophen-*d_3*/ml used as the internal standard.*

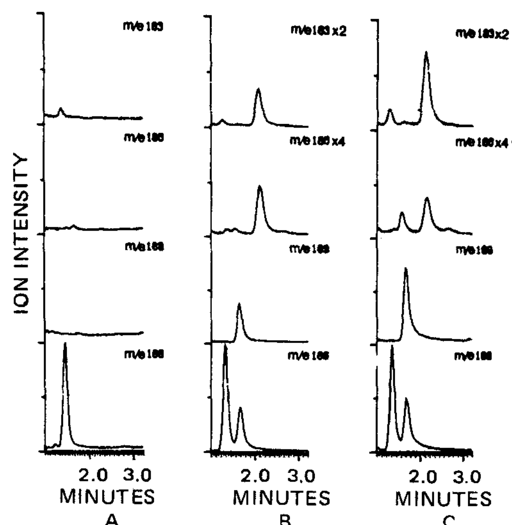


Figure 4—*Typical ion chromatograms from the determination by Procedure A of phenacetin and acetaminophen in a subject who had received an oral dose of 900 mg of phenacetin. Key: A, plasma blank, zero time; B, 6 hr after dosing, phenacetin* (m/e *180) = 17 ng/ml, phenacetin-*d_3 (m/e *183) = 25.3 ng/ml, acetaminophen* (m/e *166) = 2.51 µg/ml, and acetaminophen-*d_3 (m/e *169) = 2.52 µg/ml; and C, 8 hr after dosing, phenacetin = 6.1 ng/ml, phenacetin-*d_3 *= 25.3 ng/ml, acetaminophen = 1.62 µg/ml, and acetaminophen-*d_3 *= 2.52 µg/ml. For the purpose of display, ion tracings at* m/e *183 and 180 in B and C were multiplied by factors of two and four, respectively.*

tracted into ethyl acetate. The acetaminophen recovery for this extraction procedure (Procedure B), determined from a comparison of the peak heights of the internal standards for six separate analyses with the heights from the injection of known amounts of diazomethane-treated acetaminophen-d_3, was 89 ± 18%. Conjugated acetaminophen was calculated from the difference between the acetaminophen levels determined by Procedures A and B.

Both total acetaminophen and phenacetin can be simultaneously determined by preceding Procedure A with enzyme[18] treatment. The plasma blank, however, for phenacetin (*m/e* 180 and 183) after enzyme[18] treatment shows a number of small background ions that interfere with the accurate determination of low phenacetin levels (<5 ng/ml). In addition, the enzyme[18] treatment releases some compounds with prominent *m/e* 180 or 183 ions but with longer retention times than phenacetin. Thus, if phenacetin is to be determined in this manner, the sample injections must be spaced at least 20 min apart to ensure specificity. When using Procedure A, however, a sample can be injected every 3 min.

Human control plasma subjected to either Procedure A or B showed no significant background ions at *m/e* 169, 180, and 183. With Procedure A, a peak with a retention time shorter than that of acetaminophen was observed at *m/e* 166; ions with retention times both shorter and longer than acetaminophen were observed with Procedure B. Because of these background ions, peak heights—not peak areas—were used for the quantification of acetaminophen.

Neither phenacetin-d_3 nor acetaminophen-d_3 showed any isotope exchange when subjected to Procedure A or B.

Results obtained from the addition of known amounts of phenacetin and acetaminophen to human control plasma followed by processing according to Procedure A can be seen in Figs. 2 and 3. A least-squares analysis of the results for 12 phenacetin standards (Fig. 2) gave a slope of 0.999 ± 0.001 and an intercept of 0.304 ± 1.00 ng. The phenacetin-d_3 internal standard concentration for these analyses was 50.5 ng/ml. The corresponding curves (Fig. 3) for 12 acetaminophen standards analyzed by Procedure A and four acetaminophen standards analyzed by Procedure B gave a slope of 1.02 ± 0.015 and an intercept of 0.102 ± 0.103 ng. The acetaminophen-d_3 internal standard concentration for these analyses was 2.52 µg/ml with Procedure A and 5.00 µg/ml with Procedure B. Thus, these data suggest that the extraction, mass spectral, and GLC characteristics of the labeled and unlabeled compounds and their reactivity toward diazomethane are essentially identical.

To determine assay precision, six separate samples of control plasma, each containing 24.7 ng of phenacetin/ml and 1.31 µg of acetamino-

Table I—Plasma Levels of Phenacetin and Acetaminophen (Unconjugated, Conjugated, and Total) in a 72-kg White Male at Various Intervals after Oral Administration of 900 mg of Phenacetin[a]

Hours	Phenacetin, ng/ml	Acetaminophen, µg/ml		
		Unconjugated	Conjugated	Total
0	—[b]	—[b]	—	—[b]
1	264	5.6	2.1	7.7
2	167	6.8	6.4	13.1
3	115	6.4	7.8	14.2
4	69	5.2	9.9	15.1
5	61	4.1	8.1	12.1
6	17	2.5	9.2	11.7
7	10	2.0	9.7	11.6
8	6.1	1.6	9.3	11.0

[a] Phenacetin and unconjugated acetaminophen were determined using Procedure A; total acetaminophen was determined using Procedure B. Conjugated acetaminophen is the difference between that determined by Procedures A and B. [b] Not measurable.

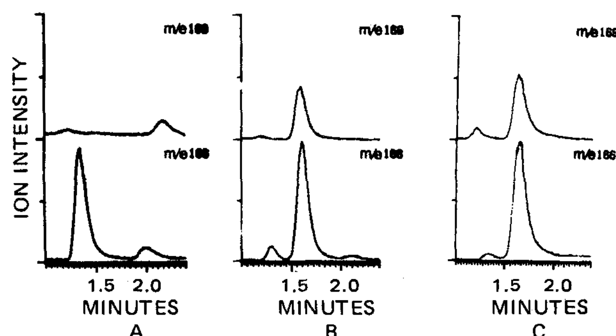


Figure 5—*Typical ion chromatograms from the determination by Procedure B of acetaminophen in a subject who had received an oral dose of 900 mg of phenacetin. Key: A, Subject 1 plasma blank, zero time; B, 6 hr after dosing, acetaminophen* (m/e *166) = 11.7 µg/ml and acetaminophen-*d_3 (m/e *169) = 5.0 µg/ml; and C, 8 hr after dosing, acetaminophen = 11.0 µg/ml and acetaminophen-*d_3 *= 5.0 µg/ml. Chromatogram A is normalized to the value of the background ions at* m/e *166 which elute before* p*-acetanisidine. The intensity of these ions relative to* p*-acetanisidine in an actual assay can be seen in B and C.*

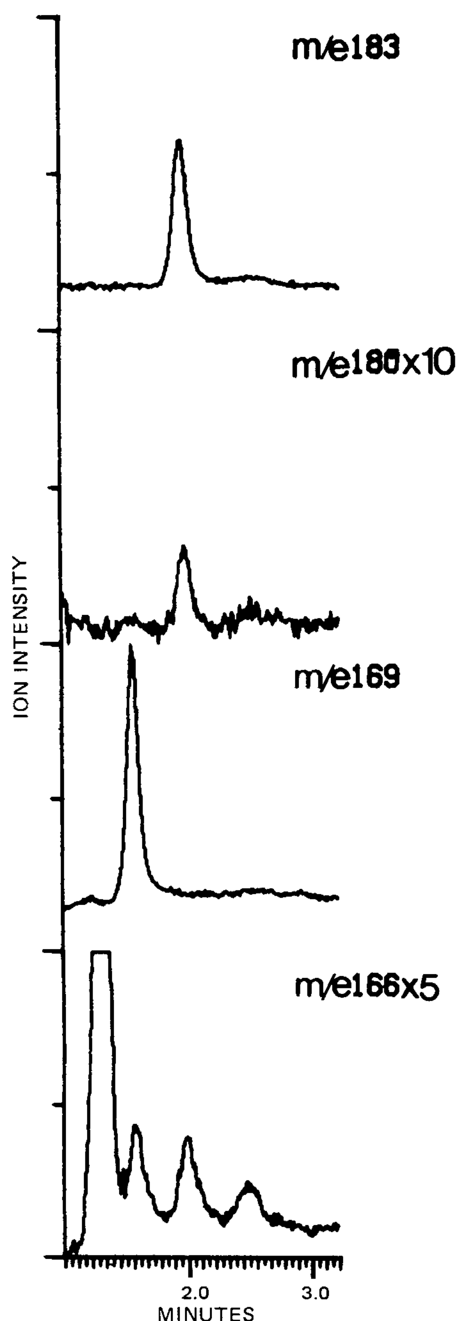


Figure 6—*Analysis of 1 ml of plasma containing 1 ng of phenacetin* (m/e *180*) *and 0.100 μg of* p-*acetanisidine* (m/e *166*) *by Procedure A. Phenacetin*-d_3 (m/e *183*) *and* p-*acetanisidine*-d_3 (m/e *169*) *internal standards were present in concentrations of 25.3 ng/ml and 2.52 μg/ml, respectively. For the purpose of display, the ion tracing at* m/e = *166 was multiplied by five while that at* m/e *180 was multiplied by a factor of 10.*

phen/ml, were analyzed using Procedure A. The average phenacetin concentration of these samples was determined to be 24.6 ± 1.0 ng/ml, while the average concentration of acetaminophen was 1.36 ± 0.08 μg/ml.

The phenacetin and unconjugated, conjugated, and total acetaminophen plasma levels for a subject who had received a 900-mg oral dose of phenacetin are reported in Table I. Typical ion chromatograms from this analysis can be seen in Figs. 4 and 5. After dosing, the phenacetin (264 ng/ml) and unconjugated acetaminophen (6.76 μg/ml) concentrations peaked at 1 and 2 hr, respectively, while the conjugated acetaminophen reached an 8–10-μg/ml plateau after 3 hr.

A least-squares analysis of the log concentration *versus* time curve for the 0–8-hr phenacetin concentrations in plasma gave a 1.3-hr elimination half-life; a similar analysis of the 3–8-hr unconjugated acetaminophen levels gave a 2.4-hr disappearance half-life. These half-lives were similar to those previously reported (23) for phenacetin and acetaminophen. Plasma levels of total acetaminophen for this subject decreased very slowly.

The sensitivity of this assay for phenacetin and acetaminophen is a function of the condition of the ion source and quadrupole rods. An analysis of a 1-ng of phenacetin/ml and a 100-ng of acetaminophen/ml plasma standard can be seen in Fig. 6. This ion chromatogram was the 113th obtained following ion source and quadrupole rod cleaning. Seven out of the available 30 μl was injected into the GLC–mass spectrometer. Both the phenacetin and acetaminophen ion peaks still had a signal-to-noise ratio greater than 5:1.

This sensitivity, while required to measure phenacetin in subjects with induced drug-metabolizing capabilities (17), should also be useful in determining basic pharmacokinetic data in humans after administration of a 300-mg therapeutic (24) phenacetin oral dose. Studies to determine the plasma concentration–time profile for phenacetin have typically required a higher than therapeutic phenacetin dose of between 1000 and 1800 mg to obtain measurable plasma levels over a sufficient timespan (23, 25–27).

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ACKNOWLEDGMENTS AND ADDRESSES

Received March 25, 1976, from the *Department of Biochemistry and Drug Metabolism, Hoffmann-La Roche Inc., Nutley, NJ 07110,* and the ‡*Department of Anesthesiology, College of Physicians and Surgeons, Columbia University, New York, NY 10032.*
Accepted for publication April 28, 1976.
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