Determination of Free Methyldopa in Plasma by High-Pressure Liquid Chromatography and Electrochemical Detection

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Abstract □ A quantitative method for the analysis of free methyldopa in plasma is described. The method is based on reversed-phase high-pressure liquid chromatography with electrochemical detection utilizing an ion-pairing agent. Separation of methyldopa from plasma is achieved by alumina adsorption. The parameters associated with the adsorption step are described. The lower limit of sensitivity is ~10 ng of methyldopa/ml with a corresponding retention time of 6.9 min.

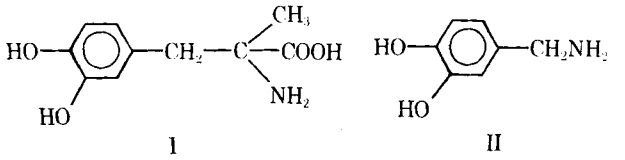
Keyphrases □ Methyldopa—high-pressure liquid chromatographic analysis using electrochemical detection, separation from plasma by alumina adsorption □ High-pressure liquid chromatography—analysis, methyldopa, plasma □ Adsorption—separation of methyldopa from plasma by alumina adsorption, high-pressure liquid chromatographic analysis

Previous measurements of small quantities of methyldopa (I) and other catechols in biological fluids have been based on separation by alumina adsorption and detection by oxidation to the fluorescent hydroxyindole derivatives. Since most catechol compounds adsorb onto alumina and form the fluorescent derivative, the approach is not specific for any one compound without additional chromatographic procedures. Kwan *et al.* (1) employed no additional chromatography and reported a mean reading for predose plasma equivalent to 280 ± 30 ng of methyldopa/ml. Subtraction of a blank value of this magnitude and variance results in considerable assay variance and severely limits sensitivity.

This paper describes a quantitative method for the determination of methyldopa in plasma that utilizes high-pressure liquid chromatography (HPLC) with electrochemical detection. Electrochemical detection previously was used successfully for detection of catecholamines (2–4). Methyldopa was separated from its metabolites and other endogenous catecholamines with reversed-phase ion-pair HPLC. 3,4-Dihydroxybenzylamine (II) was used as the internal standard.

EXPERIMENTAL

Reagents and Standards—All reagents were reagent grade or better. Water was triple glass distilled. Organic solvents were HPLC grade. The mobile phase was passed through a 0.2-μm filter[1] before introduction into the system. Pure methyldopa[2], 3,4-dihydroxybenzylamine[3], type WN-3 chromatographic alumina[3] (activity grade I), and heptanesulfonic acid[4] (sodium salt) were used. All standard solutions were made in triple glass-distilled water containing 0.005 *M* sodium metabisulfite and acidified to pH 2.6 with phosphoric acid.



I II

Instrumental Parameters—A chromatographic reciprocating pump[5], a sample injection valve[6], a 5-μm octadecylsilane column[7], a glassy carbon electrochemical detector[8], and a dual-pen recorder[9] comprised the HPLC system.

The mobile phase consisted of 0.07 *M* phosphate buffer adjusted to pH 2.6 with sodium hydroxide, 8% acetonitrile, 0.1 m*M* disodium ethylenediaminetetraacetate, and 5 m*M* heptanesulfonic acid. The anode potential was set at +0.80 v *versus* a silver–silver chloride reference electrode, and amplification was achieved with a polarographic analyzer[10]. With a mobile flow rate of 1.5 ml/min, the retention times for methyldopa (I) and 3,4-dihydroxybenzylamine (II) were 6.9 and 5.2 min, respectively.

Separation from Plasma—One milliliter of plasma was spiked with II. Then 0.1 ml of 0.05 *M* sodium metabisulfite and 0.1 ml of 5% (w/v) disodium ethylenediaminetetraacetate were added to the plasma. The plasma was adjusted to pH 7.0 by the addition of 2 ml of 0.065 *M* tris(hydroxymethyl)aminomethane buffer and then was adjusted further to pH 8.0–8.6 with 1 *M* NaOH after the batchwise addition of 135 ± 1.3 mg of acid-washed alumina (5). The sample was shaken for 15 min, and the supernate was aspirated. The alumina was washed twice with 2 ml of triple-distilled water. The methyldopa was eluted from the alumina by shaking for 15 min with 1 ml of 0.05 *M* phosphoric acid. The acid supernate was transferred immediately to a second tube from which 100 μl subsequently was injected onto the column.

DISCUSSION

Chromatography—Various parameters such as the pH and composition of the mobile phase were evaluated systematically. The ionic strength of the mobile phase greatly affected the electrode response. The effects of various ionic strengths on the bioanalytical electrodes previously were established for phosphate buffer (6) and corresponded to the present results (Fig. 1). Maximal response occurred at 0.07 *M* phosphate buffer. The effect of varying the percent composition of the organic phase in the aqueous mobile phase was described theoretically by Horvath *et al.* (7). Acetonitrile was used as the organic solvent, and the results of varying organic composition *versus* log K' (retardation factor) proved to be linear, verifying the work of Horvath *et al.* (Fig. 2).

The results of the effect of pH on K' are presented in Figs. 3 and 4. Methyldopa has a reported pKa value of 2.2 (8) for the carboxylic acid function at 25°, and K' is related directly to the ionization of the carboxylic acid functional group. The amino group with a pKa of 9.0 at 25° is ionized and is presumed to be paired with heptanesulfonic acid. Increasing the percentage of ionization of the carboxyl group decreased retention up to ~pH 3.20, where elution of methyldopa was at the solvent front. The mobile phase pH must be maintained constant since small pH fluctuations may change the chromatography significantly.

The efficiency of liquid chromatographic columns has been shown to be the greatest for compounds with K' values between 2 and 6 (9). The composition of the mobile phase was adjusted accordingly and resulted in K' values of 2.7 and 3.9 for 3,4-dihydroxybenzylamine and methyldopa, respectively.

[1] Millipore.
[2] Merck Sharp & Dohme Research Laboratories, Rahway, N.J.
[3] Sigma Chemical Co., St. Louis, Mo.
[4] Eastman Chemicals, Rochester, N.Y.
[5] Model 6000A, Waters Associates, Milford, Mass.
[6] Model 7010, Rheodyne Inc., Berkeley, Calif.
[7] Spherisorb, Altex, Berkeley, Calif.
[8] Bioanalytical Systems, West Lafayette, Ind.
[9] Fisher Scientific, St. Louis, Mo.
[10] Model 174A, Princeton Applied Research, Princeton, N.J.

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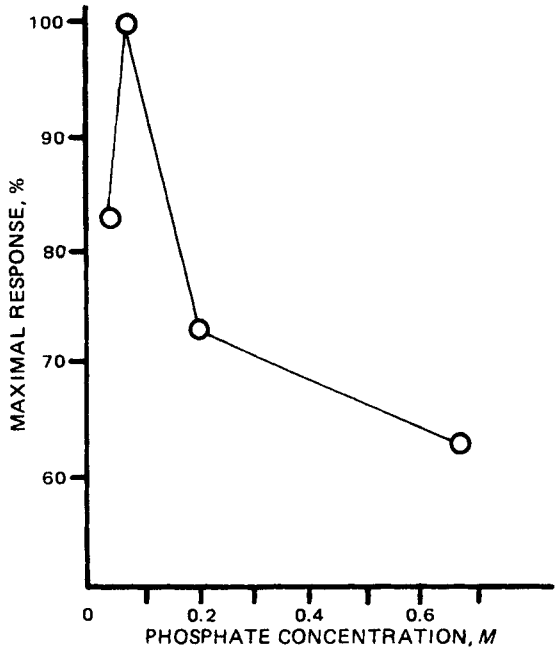

Figure 1—*Effect of ionic strength on the detector response of methyldopa. At a phosphate concentration above 0.07* M, *the current decreased in accordance with the Nernst equation for the diffusion coefficient, in which diffusion is inversely related to the ionic strength. The diminished detector response at low ionic strength is attributed to increased resistance in the electrochemical cell.*

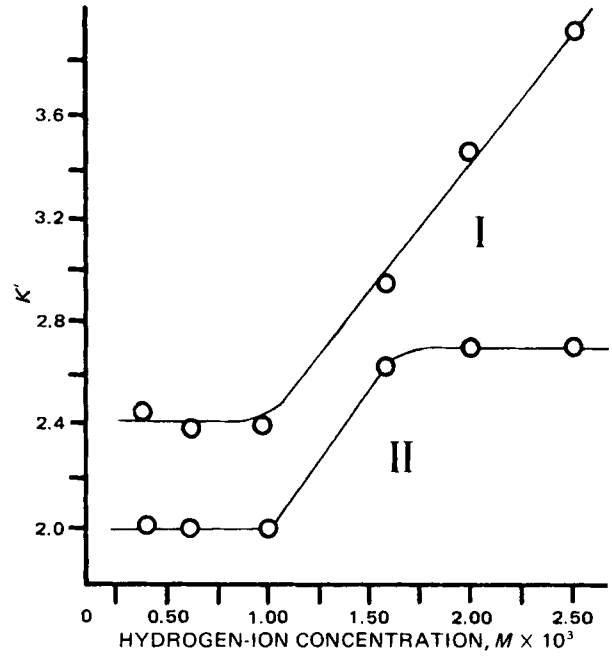

Figure 3—*Effect of hydrogen-ion concentration on the retardation factor. The mobile phase consisted of 0.1* mM *disodium ethylenediaminetetraacetate, 5* mM *heptanesulfonic acid, 0.07* M *phosphate, and 8% acetonitrile; the pH was adjusted by titration with sodium hydroxide before the organic modifier was added. The flow rate was 1.5 ml/min.*

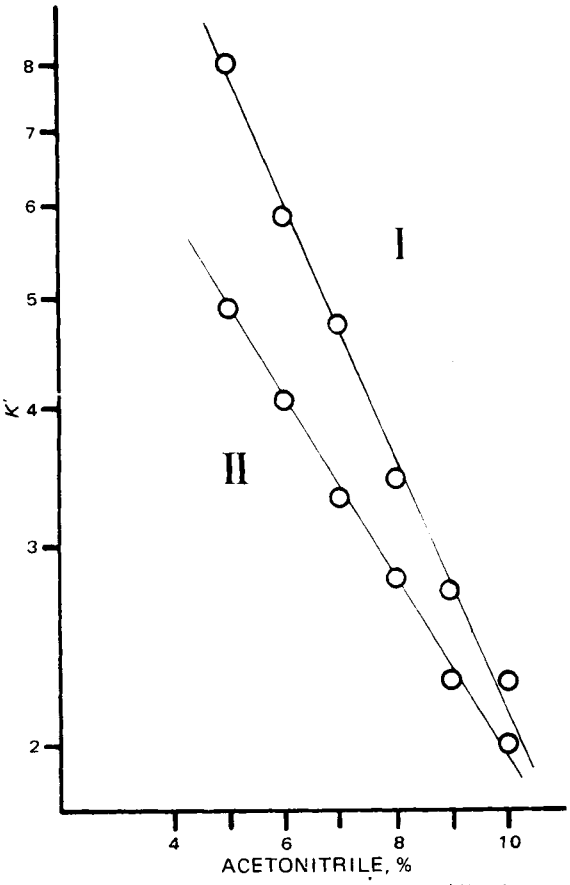

Figure 2—*Retardation factor values at varying mobile phase composition. The mobile phase consisted of 0.1* mM *disodium ethylenediaminetetraacetate, 5* mM *heptanesulfonic acid, and 0.07* M *phosphate buffer at pH 3.0 and a flow rate of 1.5 ml/min. The regression for 3,4-dihydroxybenzylamine was log y = (−0.076 ± 0.004)x + (1.066 ± 0.028), r = 0.995. The regression for methyldopa was log y = (−0.111 ± 0.044)x + (1.41 ± 0.031), r = 0.997.*

Extraction—A modified alumina adsorption method (5) was used to extract 3,4-dihydroxybenzylamine and methyldopa. The adsorption of catecholamines and methyldopa is not linear over a wide range. Both 36 and 135 mg of alumina were tried with various eluents. With 36 mg of alumina, the linear region extended to 150 ng of methyldopa/ml and saturation occurred at 600 ng/ml; 135 mg of alumina/ml produced linearity up to 400 ng/ml with saturation occurring at 750 ng/ml. Using larger

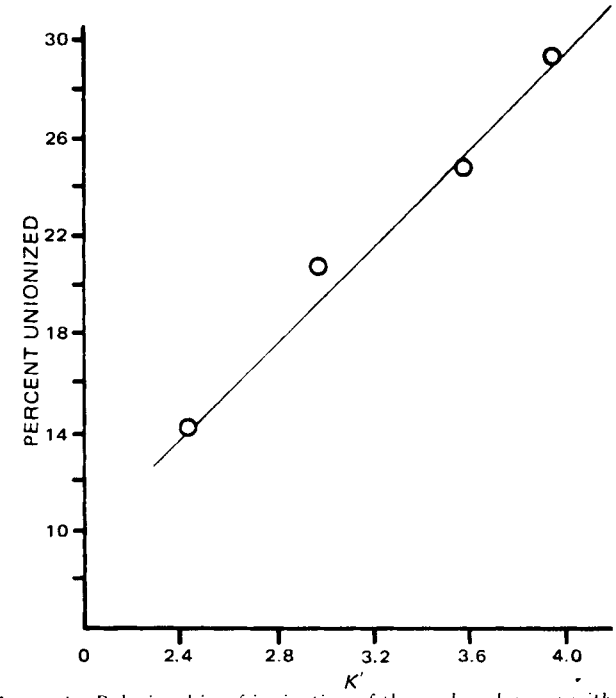

Figure 4—*Relationship of ionization of the carboxyl group with the retardation factor. The percent unionized acid function of methyldopa was calculated using literature pKa values and was not adjusted for the 8% acetonitrile in the mobile phase. The regression was* y = (9.958 ± 0.627)x + (9.490 ± 2.040), r = 0.996.

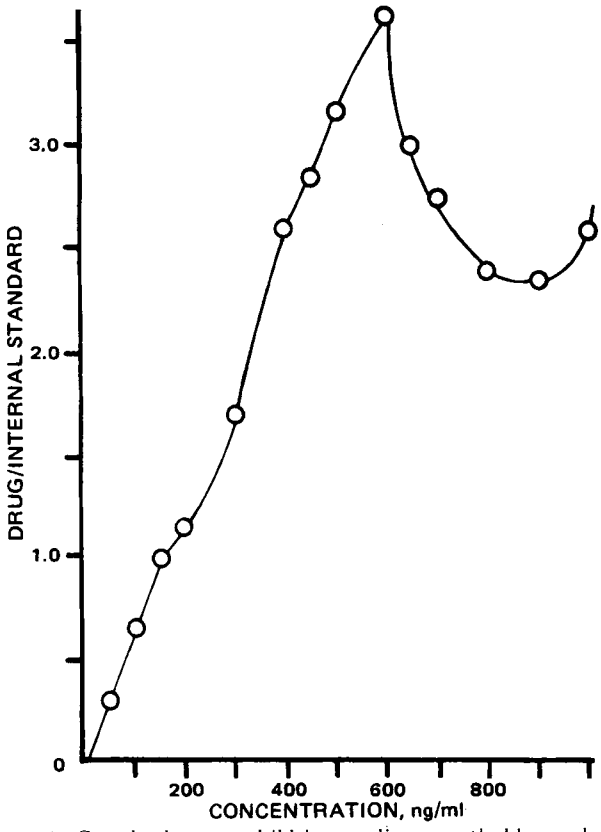

Figure 5—*Standard curve exhibiting nonlinear methyldopa adsorption. Alumina (36 mg) was used to extract methyldopa. Each value is the mean of four determinations.*

amounts of alumina was cumbersome; therefore, 1-ml plasma fractions were utilized when necessary to keep the analysis in the linear range.

The nonlinear nature of alumina adsorption was not mentioned by Kwan *et al.* (1) when measuring up to 7.5 μg of methyldopa/ml. This nonlinearity would account for substantial variation if linearity were assumed. The shape of the standard curves is typical of adsorption of drugs onto solid porous adsorbents (10) (Figs. 5 and 6).

The recovery of known quantities of methyldopa and 3,4-dihydroxybenzylamine from plasma by alumina adsorption varied for differing amounts of alumina as well as for the two drugs. The efficiency of recovery for methyldopa and 3,4-dihydroxybenzylamine was 71 and 72% with coefficients of variation of 3.8 and 3.6%, respectively, when 36 mg of alumina was used in the extraction step. The recovery efficiency changed to 84 and 56% with coefficients of variation of 2.8 and 1.8% for methyldopa and 3,4-dihydroxybenzylamine, respectively, when 135 mg of alumina was used. There were six determinations in both cases.

The linearity of the assay was demonstrated by standard curves over the 0–100- and 0–400-ng/ml ranges in which 36 and 135 mg of alumina adsorbent were used, respectively. The standard curve regressions were: $y = (0.00663 \pm 0.00011)x + (0.00520 \pm 0.00690)$, with $r = 0.999$ for 36 mg of alumina, and $y = (0.00345 \pm 0.00011)x + (0.02800 \pm 0.03100)$, with $r = 0.997$ for 135 mg of alumina, where y is the methyldopa peak height divided by the 3,4-dihydroxybenzylamine peak height and x is the concentration of methyldopa (nanograms per milliliter).

Reproducibility was demonstrated by a day-to-day coefficient of variation based on the change of the standard curve slope of 5.7% ($n = 6$) for 135 mg of alumina. The within-run coefficients of variation (drug/internal standard) were 5.4 and 1.0% ($n = 6$) for 36 and 135 mg of alumina, respectively. The lower limit of this assay with the described instrumentation and procedure (signal to noise ratio of five with no interfering peaks) is ~10 ng/ml of plasma.

Figure 7 is an example of a typical chromatogram. This assay has been applied to over 400 plasma samples and has been reliable. After ~50 assays, the electrode must be removed and the glassy carbon surface must be cleaned by mechanical polishing. Extractions are clean, and the peak resolution is very good. In the development of this assay, consideration also was given for quantitating methylnorepinephrine (III), norepinephrine (IV), and methyldopamine (V). All appeared as resolved peaks with retention times of 4.7, 4.0, and 10.6 min, respectively (Fig. 8).

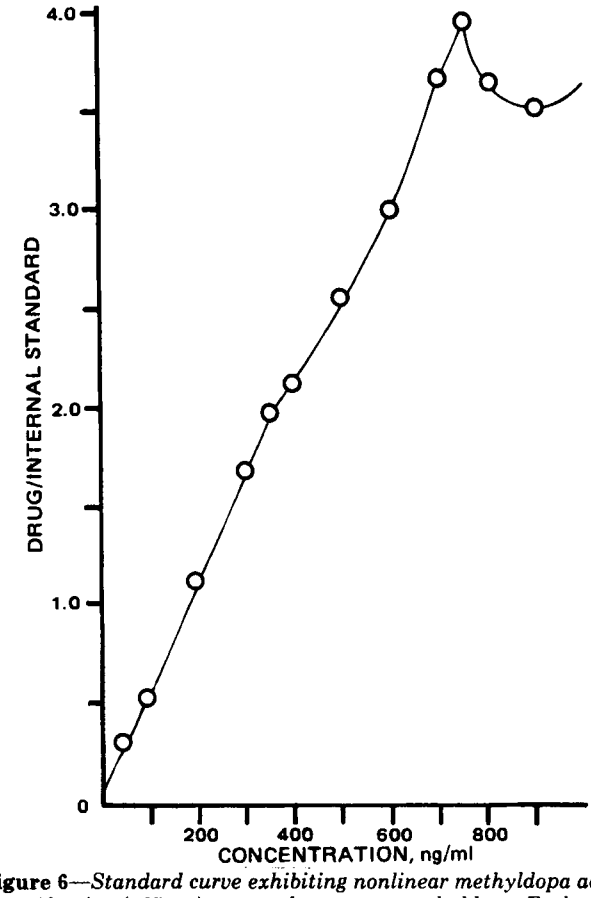

Figure 6—*Standard curve exhibiting nonlinear methyldopa adsorption. Alumina (135 mg) was used to extract methyldopa. Each value is the mean of four determinations. Note that the adsorption isotherms of both 36 and 135 mg of alumina are similar in shape, with the additional alumina shifting the curve in this figure.*

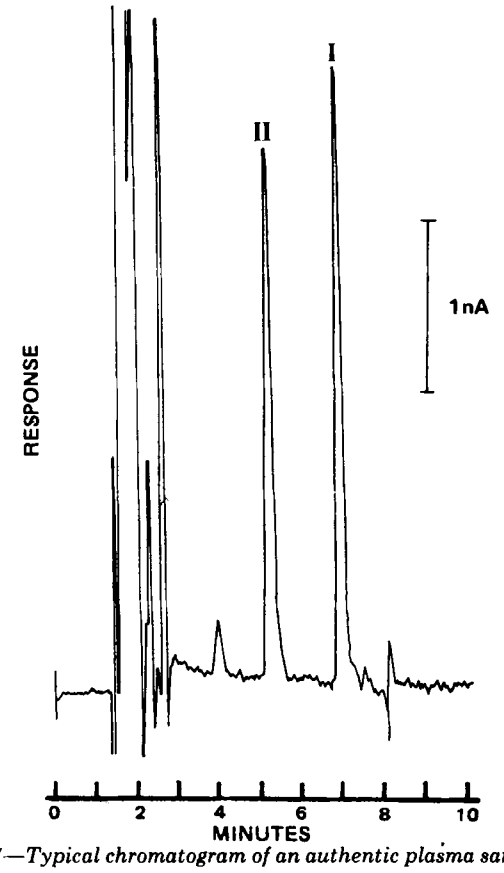

Figure 7—*Typical chromatogram of an authentic plasma sample.*

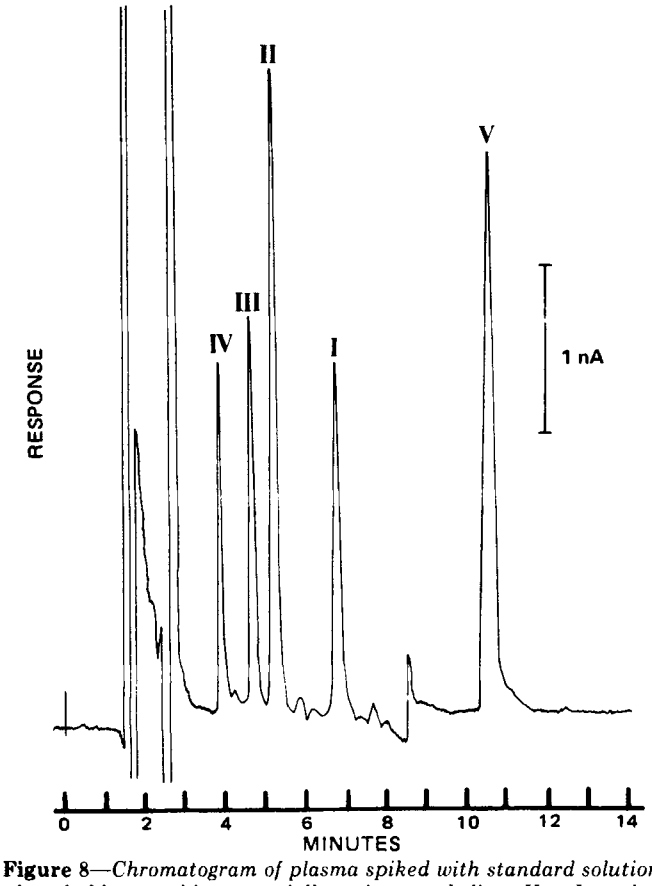

Figure 8—*Chromatogram of plasma spiked with standard solutions of methyldopa and its potentially active metabolites. Key: I, methyldopa; II, 3,4-dihydroxybenzylamine; III, methylnorepinephrine; IV, norepinephrine; and V, methyldopamine.*

Dopamine eluted with methyldopa with a retention time of 6.9 min. However, dopamine does not compromise the assay of methyldopa since normal plasma dopamine concentrations are below the sensitivity of this assay. Blank plasma samples showed no detectable interference. Changing the mobile phase to 9% acetonitrile and pH 2.40 and the ion-pair to 5 m*M* pentanesulfonic acid (sodium salt), with all other parameters remaining constant, allows complete resolution of methyldopa from dopamine. Authentic plasma samples of subjects receiving methyldopa also showed no detectable dopamine in the presence of methyldopa when resolved.

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Interactions of Aspirin with Acetaminophen and Caffeine in Rat Stomach: Pharmacokinetics of Absorption and Accumulation in Gastric Mucosa

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Received December 3, 1979, from the **National Institute of Public Health, P.O. Box 1, 3720 BA Bilthoven, The Netherlands*, and the ‡*Department of Pharmacology, Faculty of Pharmacy, State University of Utrecht, Utrecht, The Netherlands.* Accepted for publication February 27, 1980.

Abstract □ To study the pharmacokinetic interactions between aspirin (250 mg/kg) and simultaneously administered oral acetaminophen (125 mg/kg) or caffeine (50 mg/kg) in male adult rats, noninterfering GLC assays for these drugs were developed. Acetaminophen and caffeine both retarded the appearance of salicylate in plasma. During the elimination phase, acetaminophen enhanced plasma salicylate levels whereas caffeine did not. Aspirin reduced the plasma levels of both acetaminophen and caffeine during absorption and elimination. Regardless of whether the drugs had been administered separately or in combination, higher concentrations of salicylate, acetaminophen, and caffeine were found in the glandular part of the stomach compared to the nonglandular part (rumen). In both parts, the absorption of acetaminophen increased in the presence of aspirin. Simultaneous administration of aspirin with caffeine did not influence the absorption of either drug in the glandular and ruminal parts. The inhibitory action of acetaminophen and the potentiating action of caffeine on the erosive activity of aspirin are not due to any effects of these drugs on salicylate accumulation in glandular tissue.

Keyphrases □ Aspirin—administered alone and with acetaminophen or caffeine, absorption, distribution in gastric tissue, blood plasma levels, rats □ Acetaminophen—administered alone and with aspirin, absorption, distribution in gastric tissue, blood plasma levels, rats □ Caffeine—administered alone and with aspirin, absorption, distribution in gastric tissue, blood plasma levels, rats □ Pharmacokinetics—aspirin administered alone and with acetaminophen or caffeine, absorption, distribution in gastric tissue, blood plasma levels, rats

Irritation of the GI mucosa, production of erosions, and GI bleeding are serious side effects of aspirin and other nonsteroidal anti-inflammatory drugs (1, 2). Acidic anti-inflammatory drugs are thought to cause these side effects

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