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Determination of Total Salicylates, Phenacetin and/or Total *N*-Acetyl-*p*-aminophenol, and Caffeine in Biological Samples and Analgesic Formulations

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Abstract □ An analytical method was developed for the determination of phenacetin (apparent phenacetin), total salicylate (aspirin, salicylic acid, and its conjugates), total *N*-acetyl-*p*-aminophenol (*N*-acetyl-*p*-aminophenol and its conjugates), and caffeine in biological samples. Two 1-ml. samples are required. The first sample, after being acid hydrolyzed (2 *N* HCl, 2 hr., 121°) to convert total salicylate to free salicylic acid and total *N*-acetyl-*p*-aminophenol to *p*-aminophenol, is extracted with chloroform to remove caffeine and salicylic acid. *p*-Aminophenol in the aqueous phase is estimated by its reaction with phenol and sodium hypobromite. Salicylic acid extracted from the chloroform with sodium bicarbonate solution is estimated by reacting it with either Folin–Ciocalteu reagent or ferric nitrate. The caffeine, remaining in the chloroform, is estimated from its absorbance at 276 nm. In the second sample, phenacetin is hydrolyzed (8 *N* HCl, 6 hr., 121°) to *p*-aminophenol (total *p*-aminophenol), which is estimated as before. Time-dependent plasma and kidney levels of caffeine, phenacetin, total *N*-acetyl-*p*-aminophenol, and total salicylate, following the oral administration of a single dose (500 or 900 mg./kg. body weight) of aspirin–phenacetin–caffeine mixture to rats, were determined. The method also was adapted to the analysis of aspirin–phenacetin–caffeine powders.

Keyphrases □ Analgesic formulations—determination of total salicylates, phenacetin and/or total *N*-acetyl-*p*-aminophenol, and caffeine □ Plasma and tissue levels—determination of total salicylates, phenacetin and/or total *N*-acetyl-*p*-aminophenol, and caffeine □ Salicylate content, biological samples and analgesic formulations—determination, mixtures with phenacetin and caffeine □ Phenacetin and/or *N*-acetyl-*p*-aminophenol content, biological samples and analgesic formulations—determination, mixtures with aspirin and caffeine □ Caffeine content, biological samples and analgesic formulations—determination, mixtures with aspirin and phenacetin □ Aspirin–phenacetin–caffeine mixtures—determination of components in biological samples and analgesic formulations

As a result of continuing interest in the elucidation of the biochemical and toxicological mechanisms of "analgesic abuse nephropathy," different groups of researchers have employed single-entity or combination analgesics in their *in vivo* studies. Aspirin, phenacetin, and caffeine, the common components of analgesic mixtures, and their metabolites (1–6) have been studied and the current consensus points to the involvement of analgesic mixtures rather than of any single component in the production of "analgesic abuse nephropathy" (7).

In the final analysis, the availability of an analytical technique suitable for the analysis of caffeine and total amounts of salicylate and phenacetin in the tissue or biological fluid is more meaningful as injury to the tissue is related to the concentration of the injurious chemical in that tissue. This report describes an analytical method developed for the estimation of phenacetin and total *N*-acetyl-*p*-aminophenol (which includes *N*-acetyl-*p*-aminophenol and its conjugates) as *p*-aminophenol, total salicylates (including aspirin, salicylate, and its conjugates) as free salicylate, and caffeine. The major advantages of this method are its accuracy, its applicability to small size samples, and its feasibility for the analysis of aspirin, phenacetin, and caffeine in compound analgesic tablets and powders.

EXPERIMENTAL

For the GC analysis, a gas chromatograph[1] was used under the following operating parameters: column contents, 3% OV-1 on Anakrom Q, 80–100; column size, 0.42 cm. × 1.83 m. (0.125 in. × 6 ft.); column temperature, 160°; carrier gas, helium; carrier gas flow rate, 33 ml./min.; detector, flame-ionization type; injection port and detector block temperatures, 300°; hydrogen flow rate, 3 ml./min.; and air flow rate, 300 ml./min.

Chemicals—Sodium salicylate[2], *p*-aminophenol[3], phenacetin[3], and caffeine[4] of analytical grade were obtained. Analytical grade dichloroethylene, sodium bicarbonate, sodium hydroxide, dibasic potassium hydrogen phosphate, ferric chloride, and hydrochloric acid were used. Solvent grade ether and chloroform were washed successively with 1 *N* NaOH, 1 *N* HCl, and distilled water (three washings) before use. Double-distilled water was used for all purposes.

Biological Materials—The analyses were performed on rat plasma or rat kidney homogenate prepared by homogenizing (Potter-

[1] Varian 1200.
[2] Matheson, Coleman and Bell.
[3] Aldrich Chemical Co.
[4] Eastman Chemical Co., Inc.

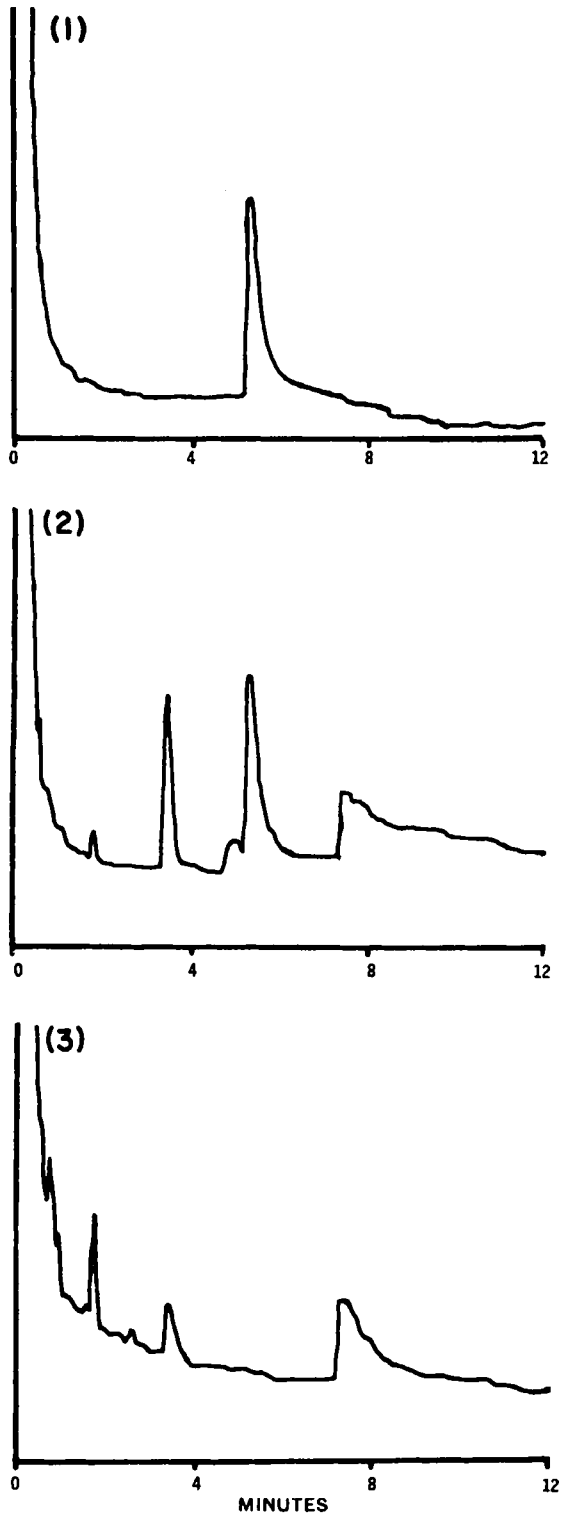

Figure 1—*GC analysis of plasma samples from control and aspirin–phenacetin–caffeine-treated rats. The chromatograms were run for 40 min., and no significant differences were observed between the control and the treated groups beyond 12 min. Key: 1, caffeine in chloroform; 2, chloroform extract of plasma from rats that received up to 900 mg. aspirin–phenacetin–caffeine/kg. (Rat Study III); and 3, chloroform extract of plasma from control group of rats.*

Elvehjem homogenizers) rat kidneys in water or 0.1 *N* HCl (1 g. wet weight/2 ml. liquid).

Analytical Method—Under suitable conditions of acid hydrolysis of biological samples containing aspirin, phenacetin, and caffeine and/or metabolites, aspirin and its metabolic products are converted to free salicylic acid; phenacetin, *N*-acetyl-*p*-aminophenol, and its metabolic products are converted to *p*-aminophenol. However, caffeine and its metabolites (demethylation and oxidation products) are not likely to undergo any change under these conditions. This knowledge, coupled with the information about the differing solubility of *p*-aminophenol, salicylic acid, and caffeine in chloroform and ether under acidic conditions, made it possible to develop the analytical technique described here.

Table I—Computer-Generated Algebraic Formulas for Calibration Curves of Salicylic Acid, *p*-Aminophenol, and Caffeine

Material	Concentration Range, mg./ml. Plasma	Equation for Calibration Curve	r^a
Salicylic acid[b]	0.0–0.52	$Y = 1.435 \times +0.016$	1.000
Salicylic acid[c]	0.0–1.50	$Y = 0.337 \times +0.006$	1.003
p-Aminophenol	0.0–0.20	$Y = 9.355 \times -0.061$	1.036
Caffeine	0.0–0.60	$Y = 0.711 \times +0.00$	1.001

[a] Regression correlation coefficient. [b] The final color reaction was developed with Folin–Ciocalteu reagent (9). [c] The final color reaction was developed with ferric nitrate (10).

When the acid-hydrolyzed sample is shaken with chloroform and the phases are separated, *p*-aminophenol is retained in the salt form in the aqueous phase while caffeine and free salicylic acid are extracted into the chloroform layer. Salicylic acid is subsequently removed with sodium bicarbonate solution; the caffeine remaining in the chloroform layer, after being thoroughly washed with sodium bicarbonate and ether, is estimated from its spectrophotometric absorption at 276 nm.

The hydrolysis is carried out for 2 hr. in 2 *N* hydrochloric acid in an autoclave at 121° under a pressure of 6.8 kg. (15 lb.)/in.^{2}. To 1 ml. of kidney homogenate or plasma in a 50-ml. centrifuge tube is added 0.67 ml. of concentrated (12 *N*) hydrochloric acid, and the volume is adjusted to 4 ml. with distilled water. After autoclaving and cooling, the mixture is shaken (for 5 min. on an automatic shaker) with chloroform (15 ml.); the phases are separated by centrifugation (5 min.) and analyzed as follows for *p*-aminophenol, salicylic acid, and caffeine.

Determination of Total *N*-Acetyl-*p*-aminophenol—To 2 ml. of the aqueous phase, transferred into a 50-ml. centrifuge tube, are added water (2 ml.), potassium hydrogen phosphate (3 g.), and ether (30 ml.) in that order. The mixture is shaken and either centrifuged or allowed to stand for 20 min. (with stopper on to minimize evaporation of ether). Then 25 ml. of the top layer (ether phase) is transferred to another 50-ml. centrifuge tube containing 5 ml. of 0.01 *N* hydrochloric acid; this mixture is shaken and centrifuged, the ether layer is aspirated, and 3 ml. of the aqueous phase is transferred to a test tube to which is added 1% phenol (1 ml.) and 1 *N* sodium hypobromite solution (1 ml.). The solution is mixed well and allowed to stand for 20 min., and its absorbance at 620 nm. is read. This method of analysis is similar to that described by Brodie and Axelrod (8).

Determination of Total Salicylate (Free Salicylic Acid)—The initial extraction with chloroform yields caffeine and salicylic acid in the chloroform phase. Ten milliliters of the chloroform phase is transferred to a 50-ml. centrifuge tube, and 5 ml. of 1% sodium bicarbonate is added. The mixture is shaken and then centrifuged, and this chloroform phase is saved for the analysis of caffeine. The aqueous phase (sodium bicarbonate solution) is used for the analysis of salicylate as follows.

An aliquot, 3.5 ml., of the sodium bicarbonate solution is transferred to a 50-ml. centrifuge tube containing 0.70 ml. concentrated hydrochloric acid, and the mixture is extracted as described below with ethylene dichloride.

After the addition of ethylene dichloride (30 ml.), the mixture is shaken for 5 min. Then it is allowed to settle and the aqueous layer is aspirated. Twenty-five milliliters of the ethylene dichloride phase is transferred to another 50-ml. centrifuge tube containing 10 ml. of 1% sodium bicarbonate or water. If sodium bicarbonate is used, the mixture is shaken and centrifuged as before. Then 3 ml. of the aqueous phase is transferred to a test tube, and 1 ml. of Folin–

Table II—Recoveries[a] of Salicylic Acid, *p*-Aminophenol, or Caffeine Added Individually to Rat Plasma

	Salicylic Acid[b]		*p*-Aminophenol[b]		Caffeine[b]	
	Milligrams Added	Percent Recovered	Milligrams Added	Percent Recovered	Milligrams Added	Percent Recovered
1	0.052	95.91	0.0165	90.91	0.120	98.91
2	0.104	92.00	0.0330	93.30	0.240	106.62
3	0.156	93.87	0.049	92.06	0.360	98.44
4	0.208	92.46	0.066	87.50	0.480	97.11
5	0.312	94.31	0.099	93.83	0.600	102.12
6	0.416	96.87	0.132	98.89	—	—
7	0.520	96.65	0.165	96.69	—	—
8	—	—	0.198	102.72	—	—
Mean ± *SE*[c]		94.58 ±0.74		94.50 ±1.69		100.62 ±1.71

[a] Calibration curves prepared with aqueous solutions. [b] Averages of at least four determinations. [c] SE = standard deviation/$\sqrt{n}$, where n is the number of determinations.

Table III—Recoveries of Salicylic Acid, *p*-Aminophenol, and Caffeine Added as Mixtures to Rat Plasma or Kidney Homogenate

Sample Number	Salicylic Acid[a]		*p*-Aminophenol[a]		Caffeine[a]	
	Milligrams Added	Percent Recovered	Milligrams Added	Percent Recovered	Milligrams Added	Percent Recovered
1	0.180[b]	—[c]	0.0385	100.40	0.072	—[b]
2	0.180[b]	—[c]	0.048	—[c]	0.384	101.5
3	0.52[b]	105.93	0.132	92.50	0.60	99.0
4	0.060	—[c]	0.124	98.71	0.060	—[c]
5	0.156	—[c]	0.0495	96.36	0.180	—[c]
6	0.113	100.00	0.054	—[c]	0.060	—[c]
7	0.180	—[c]	0.048	—[c]	0.384	102.3
8	0.42	108.26	0.100	113.15	0.60	104.3

[a] Averages of four determinations. [b] Recoveries obtained from kidney homogenate. [c] Determinations not carried out.

Table IV—Recoveries of Phenacetin as *p*-Aminophenol from Water, Rat Plasma, or Kidney Homogenate

Phenacetin Added, mg.	*p*-Aminophenol Obtained, mg.	Phenacetin Equivalent Recovered, mg.	Percent Recovery[a]
0.0300	0.0195	0.03249	108.31
0.0600	0.0398	0.06615	110.25
0.0900	0.0535	0.08884	98.70
0.1976	0.1107	0.18510	93.77
0.4200	0.2570	0.42590	101.41

[a] Average of four determinations.

Table V—Effect of Phenacetin and Salicylate on the Analysis of Caffeine in Rat Plasma

Phenacetin Present, mg.	Caffeine Present, mg.	Salicylate Present, mg.	Absorbance[a] at 276 nm.
0.152	0.600	0.000	0.448 ± 0.013[b]
0.000	0.600	0.420	0.452 ± 0.004
0.000	0.600	0.000	0.443 ± 0.003
0.152	0.000	0.000	0.016 ± 0.000
0.000	0.000	0.420	0.018 ± 0.000
0.000	0.000	0.000	0.017 ± 0.000

[a] Average of three determinations. [b] Mean ± *SE*.

Ciocalteu reagent and 1 ml. of 1.5 *N* sodium hydroxide are added in that order. The mixture is mixed well and the absorbance is read at 670 nm. after allowing it to stand for 5 min. (9). In the other method, 0.25 ml. of 1% ferric nitrate is added to the water and ethylene dichloride mixture, which is subsequently shaken and centrifuged. The concentration of salicylic acid is calculated from the absorbance of the colored aqueous phase at 540 nm. (10).

Determination of Caffeine—The 10 ml. of chloroform phase saved from the first extraction is washed (by shaking and centrifuging) twice with sodium bicarbonate solution, first with 30 ml. of 5% solution and then with 30 ml. of 1% solution. The latter is aspirated and 7 ml. of the chloroform phase is transferred to a 50-ml. centrifuge tube, evaporated to near dryness (0.1 ml.), and cooled; then 4 ml. of 2 *N* HCl solution and 30 ml. of ether are added in that order. The mixture is shaken and centrifuged, the ether phase is aspirated, and 2.5 ml. of the aqueous phase is transferred to another 50-ml. centrifuge tube to which 10 ml. of chloroform is added. The mixture is shaken and centrifuged, the aqueous phase is aspirated, and the chloroform phase is read in a spectrophotometer at 276 nm. against chloroform.

Determination of Phenacetin (Apparent Phenacetin[5])—One milliliter of plasma or homogenized kidney tissue is hydrolyzed in the presence of 8 *N* HCl (4 ml. 12 *N* acid, 1 ml. sample, 1 ml. water) by autoclaving for 6 hr. at 121° under a pressure of 6.8 kg. (15 lb.)/in.^{2}. This results in the conversion of phenacetin, *N*-acetyl-*p*-aminophenol, and its conjugates to *p*-aminophenol (total *p*-aminophenol), which is determined by the method already described except that the volume of acid hydrolysate is adjusted to 10 ml. and extracted with chloroform (30–35 ml.), and 2.50 ml. of the aqueous phase (top layer) is transferred to a 50-ml. centrifuge tube containing potassium hydrogen phosphate (4–5 g.) and water (1.5 ml.). Phenacetin in the sample is obtained by subtracting the absolute amount of total *N*-acetyl-*p*-aminophenol (determined earlier) from the absolute amount of total *p*-aminophenol (obtained after 6 hr. of hydrolysis); the resulting difference is multiplied by a factor, mol. wt. phenacetin/mol. wt. *p*-aminophenol, that includes the contribution due to the higher molecular weight of phenacetin.

Analysis of Aspirin–Phenacetin–Caffeine Powders—Three samples of such powders of unknown composition were obtained[6] and analyzed by the methods described for biological samples. However, for each type of analysis, separate calibration curves using aqueous solutions were set up. One hundred milligrams of each powder was dissolved in chloroform (100 ml.), and 1 ml. of the solution after removal of the solvent was hydrolyzed for 2 or 6 hr. under the conditions already described. The hydrolyzed solutions were analyzed for salicylate, *p*-aminophenol, and caffeine. As pro-

[5] Apparent phenacetin obtained as difference. In addition to phenacetin, this should include *N*-acetyl-*p*-aminophenol glucuronide and phenetidin present in the biological sample as a result of biotransformation.

[6] Pharmacy Research and Development Laboratories, Miles Laboratories, Inc., Elkhart, Ind.

Table VI—Plasma and Kidney Levels in Male Rats following Oral Administration of a Single 500-mg./kg. Dose of Aspirin–Phenacetin–Caffeine Powder

Hours[a]	Plasma Levels, mg./l., Mean ± SE[b]			Kidney Tissue Levels, mcg./g., Mean ± SE		
	Total Salicylate	Total APAP[c]	Caffeine	Total Salicylate	Total APAP[c]	Caffeine
0.50	237 ± 12	25 ± 1	45 ± 4	56 ± 0	28 ± 0	50 ± 11
1.00	229 ± 27	29 ± 1	36 ± 8	65 ± 0	32 ± 0	54 ± 10
1.50	285 ± 15	35 ± 1	49 ± 8	88 ± 0	37 ± 0	85 ± 10
2.00	229 ± 23	40 ± 3	41 ± 7	40 ± 0	38 ± 0	48 ± 11
4.00	206 ± 15	25 ± 3	32 ± 5	35 ± 0	28 ± 0	69 ± 16
8.00	194 ± 9	22 ± 1	38 ± 6	33 ± 0	26 ± 0	71 ± 8
24.00	140 ± 21	11 ± 2	29 ± 11	12 ± 0	21 ± 0	38 ± 11

[a] Time of sacrifice after the administration of aspirin–phenacetin–caffeine. [b] Each group comprised of nine rats; most of the values reported are the means for nine rats. [c] APAP denotes *N*-acetyl-*p*-aminophenol.

Table VII—Kidney Levels of Total *N*-Acetyl-*p*-aminophenol and Phenacetin following Acute Oral Doses of Aspirin–Phenacetin–Caffeine Powder

Hours	500 mg./kg., Mean ± SE[a]		900 mg./kg., Mean ± SE[a]	
	Total APAP[b], mcg./g.	Phenacetin, mcg./g.	Total APAP, mcg./g.	Phenacetin, mcg./g.
1.00	27 ± 1	9 ± 4	38 ± 3	26 ± 3
1.50	33 ± 3	11 ± 4	52 ± 3	46 ± 3
2.00	32 ± 1	8 ± 2	44 ± 2	11 ± 4
4.00	29 ± 2	5 ± 3	64 ± 23	26 ± 20
8.00	—	—	29 ± 2	1.5 ± 1.5

[a] Each group had three rats. [b] APAP denotes *N*-acetyl-*p*-aminophenol.

vided by the supplier, the percentage composition of all three powders was 42:42:16 of aspirin–phenacetin–caffeine.

Rat Experiments—Three studies were undertaken in which rats were employed.

Study I—In Study I*a*, male rats[7] weighing 110–150 g. were used. Rats were allowed to acclimatize to the laboratory environment until they reached the desired weight (1 week) and then they were assigned at random to different groups, nine animals in each group. Aspirin–phenacetin–caffeine (42:42:16) powder, prepared as a fine suspension in distilled water containing 0.5% sodium carboxymethylcellulose and 0.4% polysorbate 80 at a constant volume of 10 ml./kg. and at a dose level of 500 mg./kg. body weight in a single dose, was administered orally to all animals in each group. At each specified time interval following the administration of aspirin–phenacetin–caffeine, a group of rats was anesthetized; blood was obtained by cardiac puncture (opened thorax) and both kidneys were removed prior to the sacrifice of the animal. Plasma was separated from blood and stored frozen for future use; in the same way, kidneys were stored frozen after being homogenized in 0.1 *N* HCl (1 g./2 ml.). Plasma and kidney samples were analyzed for total *N*-acetyl-*p*-aminophenol, total salicylate, and caffeine at a later date.

In Study I*b*, male rats of similar weights were used and a similar protocol (three animals in a group) was followed; however, only the kidneys were removed and their phenacetin content was estimated at a later date.

Study II—Protocol was similar to the one described for Study I (three animals in a group). However, the rats, which were of a different weight range (220–260 g.), received 900 mg. aspirin–phenacetin–caffeine/kg. In addition, phenacetin was determined in both kidney and plasma samples.

Study III—About 60 ml. of plasma was obtained from control and experimental groups of male and female rats that had received an aspirin–phenacetin–caffeine mixture (42:42:16) at twice daily oral doses up to 450 mg./kg. 5 days a week for 1 month. The 1-ml. plasma samples were extracted as described for the estimation of caffeine. The final chloroform extracts from these samples were pooled separately for control and experimental groups. The volumes of these pooled samples were reduced to about 1 ml. by evaporation in a water bath, and 2-μl. samples were analyzed by GC for the detection of caffeine and/or its metabolites.

RESULTS AND DISCUSSION

Calibration curves for the estimation of salicylate, *p*-aminophenol, and caffeine in 1-ml. aliquots of rat plasma are described in Table I.

Comparison of extraction between water and plasma showed that about 95% of salicylic acid and *p*-aminophenol is extracted from plasma and quantitative extraction is possible for caffeine. Results of these studies are summarized in Table II. The determinations of either salicylic acid or *p*-aminophenol or caffeine were not interfered with when these chemicals were present either singly or as mixtures. From inspection of the results (Table III), it is apparent that the determinations of salicylic acid, *p*-aminophenol, and caffeine are consistent with the percent recoveries described here. Almost identical results were obtained for similar determinations from kidney homogenates.

After absorption of aspirin–phenacetin–caffeine-containing analgesics, the major chemical species and metabolites in plasma and tissues are likely to be: (*a*) aspirin, salicylic acid, and its conjugates (salicyluric acid and salicylic acid glucuronides); (*b*) phenacetin, *N*-acetyl-*p*-aminophenol, and its conjugates (conjugates of glucuronic acid and sulfate); and (*c*) caffeine and its metabolites (oxidation and demethylation products). Under the hydrolytic conditions described earlier, aspirin, phenacetin, and their major metabolites are converted to free salicylic acid and *p*-aminophenol. If this acid-hydrolyzed mixture is shaken with chloroform and phases are separated, the *p*-aminophenol salt is retained in the aqueous phase, whereas caffeine and salicylic acid are isolated in the lower chloroform phase. Caffeine is soluble in chloroform, but the solubility characteristics (slightly soluble) of salicylic acid in chloroform (11) made it obligatory to estimate the percent isolation of salicylic acid into the chloroform phase over the analytical concentration range. It was found that, on the average, over a wide concentration range (Table I), 95.4 ± 2% of salicylic acid[8] was extracted. Autoclaving for 2 hr. in acidic medium, which is a reported method for the hydrolysis of *N*-acetyl-*p*-aminophenol and conjugated *p*-aminophenol in biological samples to *p*-aminophenol (8), was found to be as good a method for the hydrolysis of aspirin and salicylic acid conjugates to free salicylic acid in biological samples as that described by Bedford *et al.* (5). This concentration of hydrochloric acid (2 *N*) and the duration of hydrolysis (2 hr.) were found to hydrolyze only about 5% of phenacetin. However, phenacetin was quantitatively hydrolyzed to *p*-aminophenol under more intense conditions: autoclaving for 6 hr. in the presence of 8 *N* hydrochloric acid (Table IV). The presence of salicylic acid or caffeine did not interfere with the analysis of phenacetin. Although both salicylic acid and phenacetin have significant absorption at 276 nm., neither is carried over into the final chloroform extract which is used for the estimation of caffeine. Effects of both salicylic acid and phenacetin on the estimation of caffeine were studied, and the results are summarized in Table V.

GC analyses were performed to resolve the question of whether the absorbance at 276 nm. of the final chloroform extract of biological sample obtained from animals that had received aspirin, phenacetin, and caffeine was due only to the presence of intact caffeine or included some contributions from its metabolites. The

[7] Charles River COBS-CD Strain. All animals were maintained in an animal care facility fully accredited by the American Association of Laboratory Animal Care.

[8] Mean ± *SE*.

Table VIII—Plasma and Kidney Levels of Total Salicylate, Total *N*-Acetyl-*p*-aminophenol, Phenacetin, and Caffeine following Acute Oral Administration of 900 mg./kg. Aspirin–Phenacetin–Caffeine Powder

Time[a]	Total Salicylate, Mean ± SE[b]		Caffeine, Mean ± SE		Total APAP[c], Mean ± SE		Phenacetin, Mean ± SE	
	Plasma, mg./l.	Kidney, mcg./g.	Plasma, mg./l.	Kidney, mcg./g.	Plasma, mg./l.	Kidney, mcg./g.	Plasma, mg./l.	Kidney, mcg./g.
20 min.	217 ± 11	65 ± 13	19 ± 4	36 ± 16	22 ± 1	30 ± 1	39 ± 6	31 ± 0
40 min.	270 ± 9	79 ± 6	47 ± 4	55 ± 2	29 ± 1	36 ± 0	36 ± 5	34 ± 5
1.00 hr.	244 ± 12	60 ± 5	39 ± 20	25 ± 18	34 ± 3	38 ± 3	30 ± 7	26 ± 3
1.50 hr.	271 ± 34	136 ± 22	42 ± 10	62 ± 7	38 ± 3	52 ± 4	45 ± 28	46 ± 31
2.00 hr.	256 ± 23	139 ± 20	51 ± 4	70 ± 22	28 ± 2	44 ± 2	24 ± 13	12 ± 4
4.00 hr.	281 ± 80	193 ± 53	41 ± 31	89 ± 25	33 ± 10	64 ± 23	—	—
8.00 hr.	301 ± 41	118 ± 11	83 ± 8	73 ± 7	—	—	—	—
24.00 hr.	278 ± 29	81 ± 11	68 ± 18	34 ± 11	37 ± 5	46 ± 4	6 ± 2	6 ± 2

[a] Time of sacrifice after the administration of aspirin–phenacetin–caffeine. [b] Each group comprised of three rats and the values reported represent the mean for the group. [c] APAP denotes *N*-acetyl-*p*-aminophenol.

Table IX—Analysis of Aspirin–Phenacetin–Caffeine Powders[a]

Powder	Ingredient	Percent Present	Percent Found
A	Phenacetin	42	45.25
	Aspirin	42	42.48
	Caffeine	16	15.70
Total			103.43
B	Phenacetin	42	42.59
	Aspirin	42	43.42
	Caffeine	16	15.49
Total			101.50
C	Phenacetin	42	42.44
	Aspirin	42	43.97
	Caffeine	16	14.83
Total			100.64

[a] One-milligram samples were analyzed.

results are illustrated in Fig. 1. Inspection reveals that the only additional peak in samples from treated rats not present in samples from the control group is that of caffeine. Thus, it seems that the method described is specific for the analysis of caffeine; however, if any metabolites are present in the final extract, their concentration must be negligible.

The biological usefulness of this technique is evident from the studies conducted in rats. The results of Studies I*a*, I*b*, and II are summarized in Tables VI–VIII, respectively. Inspection of Table VII reveals that within 4 hr. after the oral administration of a 500-mg. aspirin–phenacetin–caffeine mixture/kg., phenacetin disappears as a result of its conversion to *N*-acetyl-*p*-aminophenol, which is in conformity with the other reported observations (7). However, at higher dose levels of aspirin–phenacetin–caffeine (900 mg./kg.), higher levels of phenacetin are found in the kidney over a longer time following drug administration. Earlier reports (1) also demonstrated the presence of unchanged phenacetin in the dog kidney. Table VIII summarizes the results obtained at various time intervals following the oral administration of 900 mg./kg. aspirin–phenacetin–caffeine in a single dose to rats. The disappearance of the components of the aspirin–phenacetin–caffeine mixture from plasma and kidney occurs at a much slower rate at this dose level. The phenacetin concentration at 24 hr. was negligible, although significant concentrations of total salicylate, total *N*-acetyl-*p*-aminophenol, and caffeine were found in plasma and kidney.

It should be noted that: (*a*) the gentisic acid formed from salicylic acid (about 1% of the dose) and 2-hydroxyphenacetin, constituting about 1% of the phenacetin dose (4), will not be estimated by the procedure described; and (*b*) the phenacetin concentration calculated from the difference between total *p*-aminophenol (obtained after 6 hr. of hydrolysis) and total *N*-acetyl-*p*-aminophenol (after 2 hr. of hydrolysis) represents only an apparent value, because it might include the minor metabolite, *p*-phenetidin (1) and unexcreted *N*-acetyl-*p*-aminophenol glucuronide, the ether linkage of which might be resistant to 2-hr. hydrolysis. However, observations[9] indicated that *N*-acetyl-*p*-aminophenol glucuronide, if present, is completely hydrolyzed to *p*-aminophenol under the conditions described for the hydrolysis of phenacetin.

Since the method was designed to estimate caffeine, total salicylate, and total *p*-aminophenol, it can be employed for the analysis of aspirin–phenacetin–caffeine formulations. The results of the analyses of three such powders are summarized in Table IX. In comparison to currently employed column and GC techniques for the estimation of the percentage composition of aspirin–phenacetin–caffeine formulations (12), the solvent extraction method is simple and requires no equipment other than a spectrophotometer.

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[9] Under these conditions of hydrolysis, rat plasma and kidney homogenate and human urine samples obtained following the administration of phenacetin were found to be completely hydrolyzed to *p*-aminophenol.