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ACKNOWLEDGMENTS

Supported in part by National Institutes of Health Grant AM-9688.

p-Aminophenol Fluorescence and Determination in the Presence of Acetaminophen

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Received February 15, 1978, from the **Department of Chemistry, University of Cincinnati, Cincinnati, OH 45221*, and the ‡*Department of Chemistry, Wayne State University, Detroit, MI 48202.* Accepted for publication April 30, 1979.

Abstract □ The spectrophotometric and fluorometric properties of the aminophenols and of several compounds related to *p*-aminophenol were examined. A direct spectrofluorometric method for *p*-aminophenol determination at trace levels in methanol was developed and evaluated for the effect of inner filtering by acetaminophen. The method was applied to the determination of *p*-aminophenol as an impurity in acetaminophen and acetaminophen-containing tablets.

Keyphrases □ *p*-Aminophenol—analysis, spectrofluorometry, as impurity in acetaminophen tablets □ Acetaminophen—tablets, spectrofluorometric analysis of *p*-aminophenol as impurity □ Spectrofluorometry—analysis, *p*-aminophenol as impurity in acetaminophen tablets

Most methods for determining *p*-aminophenol (I) as an impurity in acetaminophen (II) involve spectrophotometric measurement of a chromophore developed *via* reaction with an appropriate reagent, either before or after chromatographic separation from II (1–10). In many of these methods, the chromophore has not been identified (4–10). Other methods involve nonaqueous titrations with perchloric acid (11) or polarographic analysis (12, 13).

To date, only an indirect fluorometric procedure for the determination of I in the presence of II has been reported (14). The fluorometric method involves a lengthy prior derivatization with benzylamine in alkaline solution. The fluorophore has not been identified.

This paper introduces a direct spectrofluorometric method for the determination of I either alone or as an impurity in II or tablets containing II. Due to overlapping UV absorption bands of these compounds, a critical evaluation of the inner filter effect was necessary. The effect of other aminophenols on the determination of I is less critical, but the possibility of positive interference in mixtures exists.

EXPERIMENTAL[1]

Solvents—Since most "spectro" quality solvents are not of suitable purity, all fluorescence work was done with purified alcohols, except where indicated. Purified alcohols were easily prepared from analytical reagent grade anhydrous alcohols by percolating each through a column packed with 30–40 cm of 40–80-mesh activated coconut charcoal[2]. The first portion (~50 ml) of the eluate from each column was highly contaminated with fluorescent impurities and was discarded. After the initial 50 ml, each column produced 5–10 liters of purified solvent before the packing was replaced.

n-Propanol did not purify as well as ethanol or methanol. Spectroquality chloroform always was used except where the chloroform fluorescence blank exceeded 3 in 100 units of full scale. To prepare fluorescent grade solvent from spectro or analytical reagent grade reagent, the chloroform was first vigorously extracted with a small volume (~10:1 v/v) of 0.45 *M* NaOH. The chloroform was recovered, washed twice with small volumes (~10:1 v/v) of water, and filtered through chloroform-wet filter paper. The resulting chloroform was suitable for spectrophotometry or spectrofluorometry.

Solutions of I—Crystals of I[3] decompose on standing and were available as black granules. Compound I was easily sublimed to gray crystals under ~4 torr at 180°; a second sublimation under similar conditions yielded white crystals with a 186–188° melting-point range (15). Alcohol or chloroform solutions, 0.01–0.001 *M*, were prepared fresh daily prior to use.

***o*-Aminophenol**—The *o*-aminophenol[3] deteriorated to black granules on standing. It was easily sublimed to pale-red crystals under ~4 torr at ~150°; a second sublimation under similar conditions yielded pale-yellow needles with a 170–174° melting-point range (16). Standard solutions were prepared fresh daily prior to use.

***m*-Aminophenol**—The *m*-aminophenol[4] was used without further purification. Although the *m*-aminophenol is somewhat more stable in alcoholic solution than the other aminophenols, standard solutions had to be prepared fresh daily.

Acetaminophen[5], Phenacetin[6], Aniline[7], Acetanilid[8], and Phenol[9]—These chemicals were used to prepare solutions without further purification.

Working Standards for Solvent Studies—All standard solutions of drugs (and related compounds) were prepared by dissolving an appropriate amount of the drug in the desired solvent and volumetrically diluting to the correct molarity. Compound I was sparingly soluble in chloroform. Standards of I in chloroform were prepared by volumetric dilutions of a concentrated methanolic I standard to the correct molarity

[1] Absorption spectra were taken on a Beckman DK-2A spectrophotometer. All fluorescence spectra were taken on a Perkin–Elmer MPF-2A spectrofluorometer.

[2] Fisher 50–200 mesh.
[3] Eastman practical grade.
[4] NF sample courtesy of Parke, Davis.
[5] NF sample courtesy of Wyeth Laboratories.
[6] American Pharmaceutical Co. USP grade.
[7] Mallinckrodt practical grade.
[8] Matheson, Coleman and Bell practical grade.
[9] Baker Chemical Co. practical grade.

0022-3549/79/1000-1306$01.00/0

Table I—Absorption and Emission Characteristics of Pharmaceuticals in Methanol

Compound	λ_A, nm	ϵ_{max}	λ_F, nm	F^a
	Fluorescent Compounds			
p-Aminophenol	235	7.9×10^3		
	300	3.7×10^3	365	100
m-Aminophenol	233	5.9×10^3		
	285	2.1×10^3	330	29
o-Aminophenol	233	6.2×10^3		
	287	3.1×10^3	330	27
Aniline	234	9.8×10^3		
	285	1.3×10^3	335	50
Phenol	230	—		
	273	1.7×10^3	297	32
Compounds Whose Fluorescence Could Not Be Characterized[b]				
Acetanilid	228	1.3×10^4	—	—
Acetaminophen	248	1.6×10^4	—	—
Phenacetin	250	1.5×10^4	—	—

[a] Based on a reading of 100 for *p*-aminophenol as an arbitrary standard. [b] In methanol, no characteristic fluorescence bands were observable.

with chloroform (the final solution was always less than 1% methanol). All synthetic samples were homogenized in a micro mill[10].

Determination of I in II—A 300-mg sample of II was accurately weighed, transferred to a 100-ml volumetric flask, and diluted to volume with methanol. A 1:100 dilution with methanol produced a solution that was retained for UV measurement of II.

The solution was inserted into the spectrofluorometer and excited at 300 nm (10-nm slit width); emission was measured at 365 nm (20-nm slit width). To minimize error from inner filtering, standards of pure (containing less than the detection limit of the method) I in the $0–12 \times 10^{-7}$ *M* range in methanol containing 3.0 mg of pure II/100 ml were prepared. To zero the instrument, a blank containing 3.0 mg of pure II/100 ml of methanol was used. A calibration plot of emission at 365 nm *versus* concentration of pure I (typically $0–12 \times 10^{-7}$ *M*) was prepared.

Determination of I in Tablets of II—For a "lot" analysis, 10 tablets of II (or the entire contents of 10 capsules of II) were weighed accurately and ground in a mortar to homogeneity. A portion of the homogeneous powder containing 300 ± 3 mg of II was weighed and transferred to a 100-ml volumetric flask, and ~75 ml of methanol was added. The solution was agitated gently for 10 min and diluted to volume with methanol. The solution was filtered through medium-speed filter paper, diluted 1:100 with methanol, and retained for fluorometric determination of I as already described. An additional 1:10 dilution of the latter solution was retained for UV measurement of II.

The UV absorbance of the solution prepared for this purpose was measured at 248 nm, and a scan of the UV spectrum from 350 to 230 nm of a representative sample for each brand of tablets or capsules was examined. If the UV scan showed additional peaks in the 300-nm region (from other drugs or additives), the sample was analyzed by another method. If the absorbance of the 1:10 dilution at 248 nm did not fall within ±0.05 unit (preferably within ±0.03 unit) of that of a 1:10 dilution of a 6×10^{-7} *M* standard containing 3.0 mg of II/100 ml, the fluorescence solution concentration was adjusted by redilution of the original sample so that its absorbance was within the desired range after a 1:10 dilution. Then the fluorescence emission was measured again as described.

RESULTS AND DISCUSSION

All aminophenols and compounds related to I contain a benzene ring; therefore, these compounds were expected to absorb intensely in the UV region (Table I). Qualitatively, the order of the molar absorptivities (ϵ_{max}) was: *p*-aminophenol > *o*-aminophenol > *m*-aminophenol > phenol > aniline.

Because the fluorescence emission process is dependent on the ability of the molecule to absorb radiant energy, it might be expected that the fluorescence emission intensity in this group of compounds would exhibit an order similar to that of the molar absorptivities. On the uncorrected spectrofluorometer, this did not appear to be the case. On that instrument, 10^{-5} *M* solutions of these compounds in methanol yielded the following order for fluorescence emission intensity: *p*-aminophenol > aniline > phenol ≅ *m*-aminophenol ≅ *o*-aminophenol.

With the assumption of a constant intensity of the xenon arc source over the small excitation wavelength range (17) and a linear photomultiplier response over the narrow emission range (18), the actual fluorescence intensity for these compounds was approximated more closely by the ratio (19) of the relative fluorescence divided by ϵ_{max}. Relative fluorescence was the emission reading for 10^{-5} *M* solutions. Reevaluation of the fluorescence emission sequence in terms of this ratio gave the following sequence: aniline > *p*-aminophenol > phenol ≅ *m*-aminophenol ≅ *o*-aminophenol.

The direct determination of any one of the fluorescent compounds in Table I would, therefore, be subject to positive interference by the presence of any of the remaining fluorescent compounds listed. The presence of large amounts of any of the last three compounds listed in Table I would cause a negative interference due to inner filtering. No error due to the possible fluorescence of the last three compounds was observed in pure methanol.

Child *et al.* (20) reported that II fluoresced at 400 nm in 1% ethanol–water but at about twice the maximum concentration used in the present study (30 μg/ml) and at 40 nm away from the 360-nm measurement using much higher signal amplification. This apparent fluorescence was not a source of error. Nang and Pitet (21) also reported fluorescence of 3×10^{-3} *M* II at 430 nm in an acidic 100% aqueous solvent, but their concentrations were much higher than those used in the present study (1.5×10^{-4} *M*) and required much higher signal amplification. Apparently, the use of methanol as a solvent reduces II fluorescence to negligible levels.

Solvent—Several other solvents were tested. Compound I did not fluoresce significantly in either water or chloroform. The small amounts of methanol did not affect the UV absorptive properties of I; and since methanol promotes fluorescence, it is more likely that the high degree of fluorescence quenching observed in chloroform was an effect of the chloroform itself rather than interference from methanol or a mixed solvent. However, I fluoresced in most common alcohols intensely enough to permit its determination in any of them (Table II). Methanol was selected because it is easily obtained and easily purified for analytical purposes. For the determination of I in II, fluorescence grade methanol must be used for all solution preparation.

Experimental Parameters—The spectrofluorometer was used because its xenon arc source provided excitation in the 300-nm region. With this spectrofluorometer, plots of the fluorescence of I *versus* concentration were linear but did not pass through the origin, indicating that a reagent blank (Fig. 1) correction was necessary. This blank effect could be seen with pure I or II in methanol. As a result, the choices of slit width, instrumental sensitivity, and excitation wavelength were critical.

With slit widths of 10 nm for excitation and 20 nm for emission, it was possible to measure 0.25% I in samples containing II with excellent sensitivity; at wider slit widths, the blank spectrum became severely distorted, making it difficult to obtain reliable blank values. At a sensitivity setting of three, the readings were more reliable than at a sensitivity setting of four, although the detection limit was lower at the latter setting. A 300-nm excitation wavelength was chosen even though the excitation maximum on the spectrofluorometer occurred at 304 nm. Excitation at wavelengths appreciably greater than 300 nm caused large variations in

Table II—Absorption and Emission Characteristics of I in Various Solvents

Solvent	λ_A, nm	ϵ_{max}	F
Water	226	5.8×10^3	
	296	1.6×10^3	4[a]
Methanol	232	7.3×10^3	
	300	2.3×10^3	48
Ethanol	301	2.5×10^3	54
n-Propanol	299	2.3×10^3	47
Chloroform	303	2.3×10^3	None

[a] Fluorescence at 375-nm emission maximum.

Table III—Evaluation of the Inner Filter Effect of II on the Determination of I in Methanol

II in Sample, mg	*A* at 300 nm[a]	Percent *IF*[b]
100	0.09	4.3
150	0.13	16
300 ± 30	0.26	18 (±3)[c]
450	0.39	33

[a] Calculated. [b] Percent $IF = (F_I + F_{I+II}/F_I) \times 100\%$, where the blank for each study contains the same milligrams of II as each sample. [c] Standard deviation = 3.3% for eight determinations on different days.

[10] Obtainable through Chemical Rubber Co., Cleveland, Ohio.

Table IV—Detection Limits of I in Pure Solution or in the Presence of II

Sample	Instrument Sensitivity Setting	Detection Limits			
		By Inspection		By Graph	
		I, %	I, *M*	I, %	I, *M*
Pure I	4	—	0.6×10^{-7}	—	0.2×10^{-7}
I in 3.0 mg % II	3	0.063	1.7×10^{-7}	0.038	1.0×10^{-7}

the blank reading while excitation below 300 nm drastically lowered the fluorescence emission intensity because of inner filtering by II and decreased excitation efficiency.

Reagent Purity and Inner Filtering—The I crystals should be white with no traces of gray. Freshly sublimed I should be stored in a tightly capped container out of the light. Moisture, air, and light tend to decompose I crystals rapidly to gray and then to black. Stock I solutions darken within several hours of preparation (~14% deterioration/day); for this reason, it is recommended that standards and samples be prepared immediately before use. Storing solutions under refrigeration in the dark slows decomposition only slightly. The filtering of solutions through medium-speed filter paper did not alter the concentration of any of the components in the samples.

If I is to be determined in the presence of II, it is important to examine the effect of the latter on the UV spectrum of the former. At II–I ratios >1000:1 (the working ratio for the analyses), the UV spectrum of the mixture actually approximated that of II alone. Thus, II should absorb an extremely large fraction of the 300-nm excitation radiation striking a typical mixture of these two compounds. Consequently, a large inner filter effect from II was observed in the direct fluorometric determination of I (Table III). Although calibration curves at various II concentrations were linear, a large variation in the magnitude of the inner filter effect was observed from day to day (Table III).

To check a sample's absorbance at 300 nm, a 1:10 dilution was made and the UV absorbance was determined at the 248-nm maximum rather than reading absorbance directly at 300 nm on the highly sloping portion

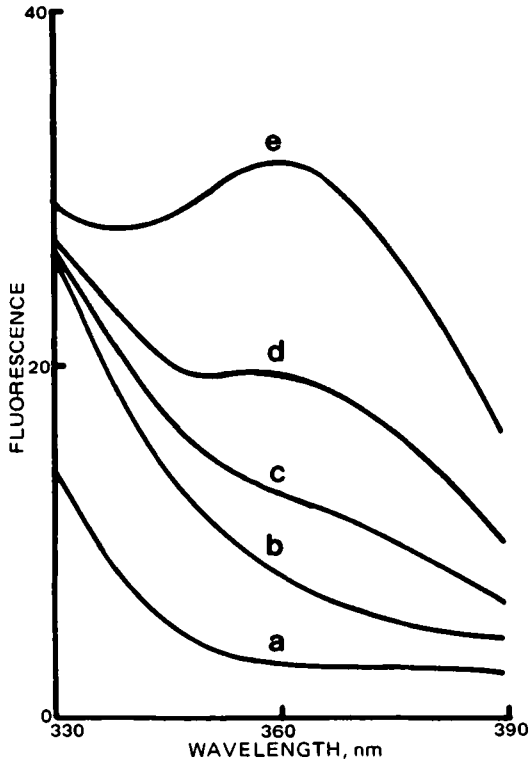

Figure 1—*Fluorescence spectra of various I concentrations in the presence of II in methanol. Key: a, methanol; b, 3.0 mg % II in methanol (blank for the determination of I in II); c, 3.0 mg % II containing 0.027 % I as an impurity in methanol; d, 3.0 mg % II containing 0.067% I as an impurity in methanol; and e, 3.0 mg % II containing 0.133% I as an impurity in methanol.*

Table V—Determination of I in Pharmaceuticals

Acetaminophen Preparation[a]	I Added[b], %	I Determined, %	Instrumental Sensitivity
Tablets 1	0.000	0.014	3
Tablets 1	0.000	0.021	4
Tablets 1	0.000	0.029	4
Tablets 2[c]	0.040	0.035[d]	3
Tablets 2	0.040	0.028	4
Tablets 3[c]	0.073	0.074	3
Tablets 3	0.073	0.067	4

[a] Tylenol tablets 2 and 3 were synthetic samples. [b] Calculated. [c] Tablet excipients had no apparent effect on the determination of I. [d] Since the amount of I is at the detection limit of I, the agreement with the expected value is not expected to be extremely good.

of the UV absorption band of II. This procedure ensured the proper content of II in the fluorescence test solution. As long as the UV absorbance of the sample solution fell within ±0.03 unit of any standard containing II (0.33 absorbance unit for 2×10^{-5} *M* II), the change in inner filter effect was negligible.

Determination of I—Compound I could be determined at low concentrations (<0.1%) in II either by inspection of the fluorescence emission spectrum (Fig. 1) or by graphical analysis. Determination by inspection only involved visual comparison of the sample fluorescence emission spectrum to those of standards and was only an estimation of one significant figure.

The detection limit of the method may not be obvious from Fig. 1. For the determination of I by inspection of spectra (Table IV), the spectrum should be scanned at medium chart speed with the scanning device at high speed to make the fluorescence band more apparent. For graphical analysis (Table IV), the fluorescence emission can be read either directly from the meter scale at the 365-nm emission peak or from the recorded spectrum using medium chart and scanning speeds (Fig. 1).

Quantitative results are shown in Table V. All commercially available II[11] tablets analyzed (Table V) contained <0.038% I in II, the detection limit of the method (Table IV). A duplicate analysis of commercially available II tablets (Tablet 1) at an instrumental sensitivity setting of four gave higher results than analysis using the same sample solutions at an instrumental sensitivity setting of three due to large variations in sample and blank readings at the former instrumental sensitivity setting (Table V).

Synthetic samples (Tablets 2 and 3) were prepared by combining known amounts of pure I with known amounts of previously analyzed tablets (Tablet 1) and then rehomogenizing in the micro mill. The results of analysis of synthetic samples were thus dependent on a "blank" correction due to II and possibly to the suspected I content of Tablet 1. For analysis at instrumental sensitivity setting four, the unspiked tablets (Tablet 1) gave higher apparent concentrations of I with large variation between replicate samples (Table V) and, therefore, much lower recoveries for the synthetic samples.

The results of the synthetic sample analyses at an instrumental sensitivity setting of three depended on a lower correction for the actual tablet content of I, and experimentally determined values compared more favorably to calculated values for the amount of I used to spike the samples. Apparent deviations from the calculated values at a lower I content (0.040%) were probably due to lower precision in both the determination of low percent of I and in the actual tablet content that falls below the detection limit for the method rather than other possible sources of interference (*e.g.*, insoluble excipients). This speculation is supported by the small positive error in the analytical results where the content of I was higher (Tablet 3).

CONCLUSION

Since no TLC or column chromatography was used, there was no loss of I during the analysis as is possible in methods involving separations (22). Also, there was no possibility of an incomplete or competing chromophore- or fluorophore-forming reaction. This fact decreased the possibility of formation of interfering species or of the decomposition of I. Most methods for the determination of I are quite sensitive, but many specify no detection limit for I alone or in combination with II (1, 3, 10, 16, 23, 24). Many methods that do specify detection limits, expressed as percent I in II, possess sensitivities that are not lower than the direct

[11] Tylenol preparation containing 500 mg/tablet, McNeil Laboratories.

fluorometric method (1, 10, 14, 22, 24). Other methods were specifically designed for the determination of I in biological specimens (1, 10) or for the determination of II and related compounds after conversion to I (1, 10, 14).

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ACKNOWLEDGMENTS

The authors thank Wayne State University and the Wayne County Medical Examiners Office for the use of their facilities.

Comparative Pharmacokinetics of Coumarin Anticoagulants XLII: Effect of Phenobarbital on Systemic Availability of Orally Administered Dicumarol in Rats with Ligated Bile Ducts

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Received February 22, 1979, from the *Department of Pharmaceutics, School of Pharmacy, State University of New York at Buffalo, Amherst, NY 14260.* Accepted for publication April 19, 1979.

Abstract □ The purpose of this investigation was to determine if the previously demonstrated inhibitory effect of phenobarbital treatment on the systemic availability of orally administered dicumarol in rats is related to the known effect of phenobarbital on bile output. It was found that phenobarbital had no apparent effect on the systemic availability of an aqueous dicumarol suspension in rats with ligated bile ducts. Compared to results obtained previously on normal rats, bile duct-ligated rats absorbed and eliminated dicumarol much more slowly and absorbed much less of the anticoagulant. On the other hand, the relative inductive effect of phenobarbital treatment on dicumarol elimination was similar in normal and in bile duct-ligated animals. The latter exhibited substantial serum transaminase elevations, indicative of liver damage presumably secondary to cholestasis. These results demonstrate that a drug–drug interaction can depend markedly on the pathophysiological status of the animals.

Keyphrases □ Phenobarbital—effect on dicumarol systemic availability in bile duct-ligated rats □ Dicumarol—phenobarbital effect on systemic availability in bile duct-ligated rats □ Anticoagulants—dicumarol, systemic availability, effect of phenobarbital in bile duct-ligated rats □ Bile duct—ligation, effect on rats, dicumarol systemic availability, phenobarbital □ Drug interactions—phenobarbital effect on dicumarol systemic availability in bile duct-ligated rats

The systemic availability of orally administered dicumarol in humans (1) and rats (2) is reduced by pretreatment of the subjects with a barbiturate. Oral phenobarbital sodium administration, 75 mg/kg, for 5 days before and 2 days after oral administration of dicumarol suspension to rats reduced the systemic availability of the anticoagulant from 84 ± 8 to 48.7 ± 10% (mean ± *SD*). Similar effects were observed when phenobarbital was administered intravenously (2).

Since phenobarbital treatment increased bile output (3), the inhibitory effect of phenobarbital on dicumarol absorption possibly is mediated by complexation of the anticoagulant with bile salts or by altered GI motility caused by increased bile flow. A study was initiated to determine if phenobarbital treatment affects systemic dicumarol availability in rats with ligated bile ducts.

EXPERIMENTAL

The experimental procedures were described previously (2). Briefly, adult male Sprague–Dawley rats weighing ~300 g received daily oral doses of phenobarbital sodium, 75 mg/kg, or the same volume of saline solution for 5 days. Their right jugular vein was cannulated on the 5th day to facilitate frequent blood withdrawal. In the morning of the 6th day, the rats received an intravenous tracer dose of ^{14}C-dicumarol by rapid injection and 50 mg of dicumarol/kg in aqueous suspension by gastric tube.

Blood samples were collected periodically, and daily phenobarbital treatment was continued until the end of the experiment. Food, but not water, was withdrawn for 24 hr, starting 12 hr before dicumarol administration. Plasma was assayed for ^{14}C-dicumarol and unlabeled dicumarol. The ratio of areas under the concentration–time curves for the labeled and unlabeled drug, normalized for dose, was used to calculate systemic

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