moved by filtration. The filtrate was concentrated to 600 ml. under vacuum at 60° and 6 l. acetone was added resulting in crystallization of crude V*a*. The compound was dissolved in 1 l. water and extracted with 300 ml. chloroform. The pale yellow, aqueous layer was partially decolorized with charcoal and the solution was concentrated to 300 ml. under vacuum at 50°. The solution was diluted with 400 ml. ethanol and the resulting white crystalline V*a* was isolated by filtration. The compound was recrystallized from a mixture of 500 ml. hot (80°) water by addition of 500 ml. ethanol. The product was isolated by filtration giving 95 g. (50%), m.p. 223–225° (dec.). Cellulose TLC (Solvent B) and analytical ion-exchange chromatography showed no impurities.

Anal.—Calcd. for $C_{18}H_{35}N_2O_9SP$ (486.52): C, 44.43; H, 7.25; N, 5.76; P, 6.37; S, 6.59; eq. wt., 486.52. Found: C, 44.70; H, 7.40; N, 5.90; P, 6.50; S, 6.60; eq. wt., 492.; H_2O, 3.15 (analyses corrected for 3.15% H_2O); $[\alpha]_D^{29}$ + 120° (H_2O).

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Determination of Phenethanolamine Drugs in Biologic Specimens by Ultraviolet Spectrophotometry

JACK E. WALLACE

Abstract □ A rapid differential method for spectrophotometrically determining ephedrine and certain related compounds in biologic specimens in the presence of other alkaline drugs is presented. In the procedure, the compounds are oxidized by means of alkaline periodate to benzaldehyde which, in comparison to the original compounds, has a much higher molar absorptivity for UV radiations. In order to enhance significantly the sensitivity and specificity of the procedure benzaldehyde is subsequently converted to its semicarbazone derivative. Only compounds that have a benzyl alcohol functional group and are extractable as an alkline drug can be analyzed by the method. The procedure is sufficiently sensitive to permit analysis of the compounds in biologic specimens after therapeutic doses.

Keyphrases □ Phenethanolamine in biological specimens—determination □ Periodate oxidation—phenethanolamine determination □ GLC—identification □ IR spectrophotometry—identification □ UV spectrophotometry—analysis

The extensive use of phenethanolamine compounds in pharmaceutical preparations has led many investigators to develop rapid and sensitive procedures for determining these compounds in biologic and pharmaceutical specimens. The many published methods attest not only to the importance of the drugs but also to the difficulties inherent in their analysis. Ephedrine (1-phenyl-2-methylamino-propanol) and the related compounds phenyramidol, pseudoephedrine, and phenylpropanolamine have benzenoid spectra which are neither sensitive nor characteristic in their absorption of UV radiations. Methods which rely on direct UV techniques, therefore, have little application to the analysis of these compounds in biologic systems (1, 2).

The colorimetric procedures (3–5) for determining ephedrine-type compounds also give positive reactions with many other alkaline-extractable drugs. The fluorometric methods (6) detect submicrogram quantities but require specialized instrumentation and are often time-consuming. The problems in applying gravimetric or nonaqueous titration systems (7) to the analysis of compounds in biologic specimens are evident. Paper (8) and gas chromatographic (9, 10) procedures can be made very sensitive, but they do not provide the forensic scientist with specific information concerning the molecular structure of the unknown compounds. None of the previously mentioned methods are capable of screening large numbers of samples in a short time period which is often necessary in the clinical laboratory.

Using periodate oxidation, Heimlich (11) developed a specific analytical technique for determining phenylpropanolamine in urine. Derivative formation by his method affords a sensitivity three to four times greater than that for direct spectrophotometric determination of unconverted phenylpropanolamine. Periodate oxidation was also used by Chafetz (12) for the assay of several phenethanolamine compounds in pharmaceutical preparations, but the procedure is not applicable to the analysis of the drugs in biologic specimens.

The use of steam distillation and a dichromate reagent to obtain benzaldehyde from ephedrine was reported

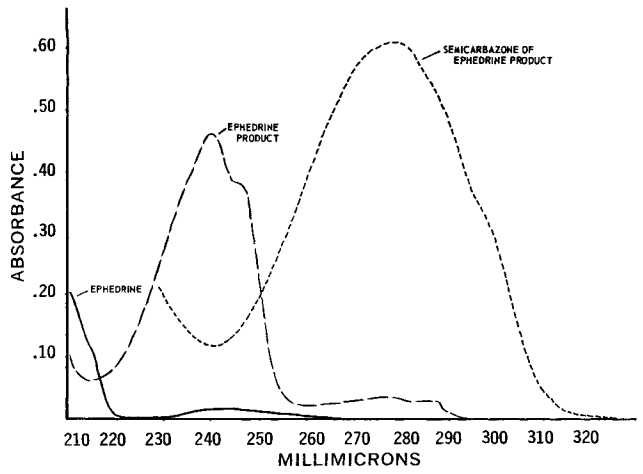

Figure 1—*UV absorption spectra of ephedrine in water, of the ephedrine product in* n*-hexane, and of the semicarbazone of the ephedrine product in water, each corresponding to an ephedrine concentration of 5 mcg./ml.*

previously (13). After conversion to a semicarbazone, the aldehyde imparted to the method a sensitivity approximately 10 times greater than that of the Heimlich procedure. For determining the drug in biologic fluids the steam distillation method is adequate, but the requirement for 50 ml. of distillate to ensure quantitative results is a major disadvantage. In addition, the need for a constant source of steam makes it difficult to perform a number of tests simultaneously.

In the method of this report, periodate is used to oxidize the phenethanolamine compounds to benzaldehyde. The procedure is rapid, group specific, and more sensitive than any of the previously reported procedures. Preliminary separation of the phenethanolamine compounds from other types of alkaline drugs is not required.

EXPERIMENTAL

Instrumentation—UV absorption measurements were made using a Beckman DK-2A ratio-recording spectrophotometer with linear presentation. A Beckman IR-9 spectrophotometer was used for IR absorption measurements. A Barber-Colman model 5000 gas chromatograph with a U-shaped 1.83-m. (6-ft.) 2% QF-1 column was used for gas chromatographic analysis.

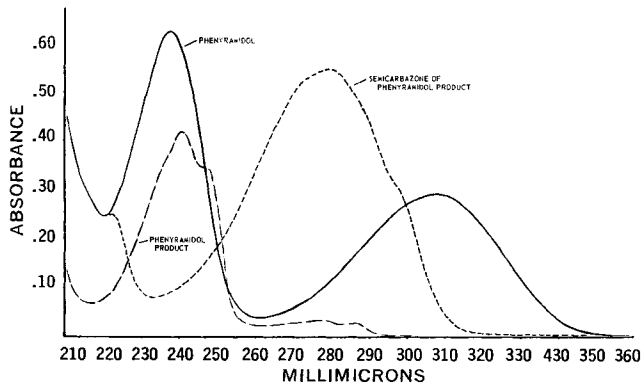

Figure 2—*UV absorption spectra of phenyramidol in water, of the phenyramidol product in* n*-hexane, and of the semicarbazone of the phenyramidol in water, each corresponding to a phenyramidol concentration of 10 mcg./ml.*

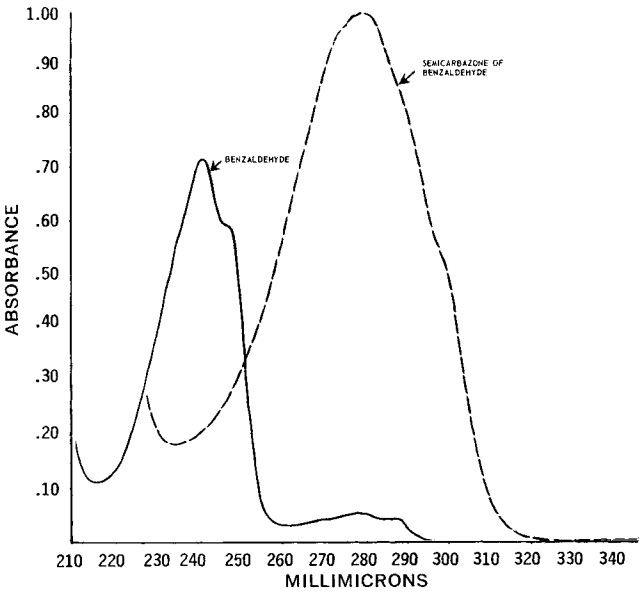

Figure 3—*UV absorption spectra of benzaldehyde in* n*-hexane and of benzaldehyde semicarbazone in water, each corresponding to a benzaldehyde concentration of 5 mcg./ml.*

Method—Volumes of 5 to 10 ml. of blood, serum, or urine are placed in a 250-ml separator and made strongly alkaline by the addition of 1 *N* NaOH. Five- to ten-gram amounts of tissue are homogenized and treated in a like manner. One hundred fifty milliliters of anhydrous ether is added and the mixture is shaken vigorously for 3 min. The ether is separated from the aqueous phase by filtration through a fast flowing filter paper. Complete recovery of the ether should not be attempted, but the volume of recoverable ether is immediately recorded and the loss included in the final calculations. Ten milliliters of 0.5 *N* hydrochloric acid solution is added to the residual ether and the mixture is shaken vigorously in a separator for 3 min. Nine milliliters of the aqueous layer is transferred to a 250-ml. round-bottom flask with a ground-glass neck for attachment to a water-cooled reflux condenser. To the flask is also added 2 ml. of a 4% solution of periodic acid (G. Frederick Smith Chemical Co., Item No. 74 or equivalent), 9 ml. 1 *N* NaOH, and 50 ml. of spectroquality grade *n*-hexane (Fisher No. 334 or equivalent).

The contents of the flask are refluxed, using a Glas-Col heating mantle (Glas-Col Apparatus Co., Inc., Terre Haute, Ind.) supplied with 40 v. through a variac. The slow reflux is maintained for 30 min. with constant magnetic stirring of the flask contents. After cooling, the *n*-hexane layer is removed from the flask by means of a transfer pipet, and subsequently washed with 10 ml. of 0.5 *N* HCl. Approximately 3 ml. of the organic solvent is transferred to a cell and its absorption from 220–340 mμ determined in a recording spectrophotometer against a blank solution of *n*-hexane. If analysis at a single wavelength is required, determine the absorption at 240 mμ.

The sensitivity and specificity of the procedure can be significantly enhanced by converting the carbonyl product to its corresponding semicarbazone. This is accomplished by reacting the product in 4–40 ml. of *n*-hexane (depending upon the amount of absorbance observed at 240 mμ) with 4 ml. of 0.05 *M* semicarbazide hydrochlo-

Table I—Standard Curve Data of Ephedrine Reaction Product

Ephedrine in Sample, mg./ml.	Absorbance of Reaction Product[a]	Absorbance Concentration	Absorbance of Semicarbazone of Reaction Product[b]	Absorbance Concentration
10.0	0.890	0.089	1.23	0.123
8.0	0.718	0.090	0.994	0.124
6.0	0.552	0.092	0.751	0.125
4.0	0.356	0.089	0.491	0.123
2.0	0.190	0.095	0.246	0.123

[a] In hexane. [b] In aqueous semicarbazide.

Table II—Recovery Studies of Ephedrine

Ephedrine Added, mcg./ml.	Recovery[a], Mean ± SD, mcg./ml—— Blood	Urine	Homogenized Tissue
10.0	8.72 ± 0.10	9.43 ± 0.15	8.37 ± 0.11
5.0	4.31 ± 0.05	4.72 ± 0.10	4.34 ± 0.20
2.5	2.15 ± 0.03	2.28 ± 0.04	[b]
Av. recovery, %	86.5	93.3	85.3

[a] Determined from semicarbazone derivative. [b] Not determined.

ride solution buffered to pH 4.0 with sodium acetate. The reaction is conveniently done at room temperature in a flask connected to a vacuum rotary evaporator. Semicarbazone formation is complete when the *n*-hexane has been removed. The final aqueous solution is read at 278 mμ against a 0.05 *M* semicarbazide blank. A calibration curve for the analysis is prepared from appropriate aqueous solution of the drug oxidized directly by the alkaline periodate reagent in the presence of *n*-hexane.

RESULTS

Ephedrine and several related phenethanolamine compounds give reproducible amounts of aryladehyde when oxidized with an alkaline periodate reagent. The UV absorption spectra of ephedrine, its reaction product, and the reaction product's semicarbazone derivative are shown in Fig. 1. With the exception of absorbance per unit concentration of drug, identical data are obtained for pseudoephedrine and phenylpropanolamine. Unoxidized phenyramidol has a different UV absorption from that of ephedrine, yet the same spectral curves are observed after oxidation (Fig. 2). Each of the reaction products has an absorption maximum at 240 mμ in *n*-hexane, and a 278 mμ maximum when converted to their respective semicarbazones. The UV spectra of benzaldehyde and its semicarbazone are shown in Fig. 3. It is apparent that the carbonyl products from the phenethanolamine compounds are benzaldehyde. Gas chromatographic and IR data also support this observation. In the analysis of each drug a linear relationship over the range 0 to 20 mcg./ml. is obtained (Tables I and III).

Table II demonstrates the capability of the procedure to recover ephedrine from biologic specimens to which the drug has been added. In each case the average recovery was greater than 85%. Numerous alkaline and neutral drugs that are extracted with ephedrine in a general toxicologic analysis were investigated for possible interference (Table III). It is desirable after the reaction with periodate to wash the *n*-hexane with 0.5 *N* HCl. Those alkaline drugs which do not have the phenethanolamine group are not oxidized to the corresponding aldehyde; consequently, they are extracted from the organic solvent layer with dilute acid. In addition, the unoxidized drugs are generally incapable of reaction with semicarbazide.

Table III—Compounds Investigated for Interference with the Determination of Ephedrine[a]

Compound	Absorbance of Product[b]	Absorbance of Product Semicarbazone[c]	Compound	Absorbance of Product[b]	Absorbance of Product Semicarbazone[c]
Ephedrine	1.78	2.47	Chlordiazepoxide	0.07	0.01
Pseudoephedrine	1.78	2.47	Chlormezanone	0.04	0.01
Phenylpropanolamine	1.93	2.75	Chloroquin	0.02	0.03
Phenyramidol	0.84	1.10	Chlorthen	0.05	0.01
Blank	0.02	0.01	Chlorpheniramine	0.05	0.01
Acetophenazine	0.02	0.01	Chlorphentermine	0.08	0.01
Amitriptyline	0.07	0.02	Chlorpromazine	0.09	0.01
Amphetamine	0.07	0.01	Chlorprothixene	0.08	0.02
Antazoline	0.06	0.03	Clemizole	0.06	0.01
Atropine	0.07	0.01	Cyclizine	0.09	0.01
Azacyclonol	0.09	0.03	Danthron	0.13	0.03
Benactyzine	0.04	0.01	Dextromethorphan	0.06	0.01
Betamethasone	0.04	0.01	Diazepam	0.06	0.02
Bisacodyl	0.06	0.01	Diethylpropion	0.06	0.02
Bishydroxycoumarin	0.06	0.01	Diphenhydramine	0.07	0.01
Bromodiphenhydramine	0.08	0.01	Diphenylpyraline	0.07	0.01
Brucine	0.06	0.01	Doxylamine	0.06	0.01
Caffeine	0.02	0.01	Ergotamine	0.06	0.01
Cantharidin	0.06	0.01	Ethinamate	0.04	0.01
Captodiamine	0.19	0.07	Ethoheptazine	0.04	0.01
Carbinoxamine	0.06	0.01	Glutethimide	0.02	0.01
Carisoprodol	0.04	0.01	Hydroxyzine	0.04	0.01
Imipramine	0.06	0.02	Pipenzolate	0.02	0.01
Isocarboxazid	0.15	0.05	Piperidolate	0.07	0.01
Isoniazid	0.06	0.01	Prochlorperazine	0.03	0.01
Lidocaine	0.04	0.01	Procyclidine	0.06	0.01
Mepenzolate	0.03	0.01	Promethazine	0.03	0.02
Meperidine	0.07	0.01	Propoxyphene	0.03	0.01
Mephentermine	0.09	0.01	Pyrathiazine	0.03	0.02
Meprobamate	0.04	0.01	Pyrrobutamine	0.04	0.04
Methamphetamine	0.05	0.01	Quinidine	0.06	0.01
Methapyrilene	0.06	0.01	Reserpine	0.04	0.01
Methoxyphenamine	0.06	0.01	Salicylic Acid	0.06	0.01
Methylphenidate	0.05	0.01	Strychnine	0.07	0.01
Methyprylon	0.06	0.01	Sulfadiazine	0.07	0.01
Oxazepam	0.16	0.04	Thenyldiamine	0.02	0.02
Pargyline	0.06	0.01	Thioridazine	0.06	0.01
Phenaglycodol	0.23	0.22	Thonzylamine	0.04	0.01
Phenelzine	0.09	0.03	Tranylcypromine	0.13	0.18
Phenindamine	0.05	0.02	Trimethobenzamide	0.03	0.01
Phenmetrazine	0.07	0.01	Tripelennamine	0.02	0.01
Phenylephrine	0.02	0.01	Triprolidine	0.06	0.01
Phenyltoloxamine	0.04	0.01	Tubocurarine	0.06	0.01
Pilocarpine	0.05	0.01	Yohimbine	0.06	0.01

[a] Each compound was added directly to the reaction flask. The level of each corresponds to a concentration of 20 mcg./ml. in *n*-hexane as well as in aqueous semicarbazide. Each value is the average of three determinations. [b] Read at 240 mμ. The *n*-hexane was washed with 0.5 *N* HCl prior to determination. [c] Read at 278 mμ against a semicarbazide blank.

Table IV—Ephedrine Distribution in the Rat[a]

Tissue	Distribution Ratio[b], Mean ± *SD* Concn. in Tissue/Concn. in Liver
Blood	0.13 ± 0.03
Brain	0.38 ± 0.05
Fat	0.02 ± 0.00
Kidney	2.21 ± 0.28
Liver	1.00
Lung	1.20 ± 0.27
Muscle	0.29 ± 0.04

[a] Rats were fed *via* stomach tube 25 mg. of ephedrine per kg. body weight. [b] Average of 10 rats.

To determine the pattern of distribution into the various tissues, rats were fed, *via* stomach tube, 25 mg. of ephedrine (as the free base) per kilogram of body weight. Levels of the drug found in blood and tissues in decreasing order are: kidney, lung, liver, brain, muscle, blood, and fat. Average distribution ratios for 10 rats are given in Table IV.

DISCUSSION

The ability of alkaline periodate to cleave carbon-to-carbon bonds of 1,2-glycols and α-aminoalcohols, provided that the amine is primary or secondary, is the basis for the procedure of this report. Removing the benzaldehyde from the aqueous oxidation phase by continuous extraction with *n*-hexane allows the reaction with the phenethanolamine compounds to proceed to stoichiometric completion. This adaptation evidently accounts for the increased sensitivity of the proposed method over that of techniques described previously. Of interest is the lack of any UV-absorbing products in the hexane layer after oxidation of certain phenolic compounds (Table III). Phenolic metabolites such as *p*-hydroxyephedrine are not extracted into the hydrocarbon because of molecular polarity differences. Therefore, interference from the many types of phenolic amines normally present in biologic specimens is also eliminated. It is apparent that periodate oxidation of certain phenethanolamine compounds yields only one compound sensitive to ultraviolet radiations, benzaldehyde (Figs. 1–3).

The high sensitivity of the proposed method can best be illustrated by comparing the slopes of standard curves associated with several published procedures. Heimlich *et al.* (11) reported, in 1-cm. cells, a slope of approximately 0.008 absorbance units per microgram of phenylpropanolamine. Using mandelic acid as a test compound for periodate oxidation, Chafetz (14) obtained a slope of approximately 0.09, which approaches the order of sensitivity obtained with the method of this report. The slope of the standard curve for dichromate oxidation of ephedrine (14) was 0.058 for the product and 0.11 for its semicarbazone. The slopes of the standard curves in the proposed procedure which are 0.09 and 0.12 for the aryladehyde product and its semicarbazone, respectively, compare favorably with the sensitivities obtained by other investigators using UV spectrophotometry.

In the dichromate method, as well as other available spectrophotometric techniques, an almost prohibitive amount of manipulation is required when applying the procedures to the analysis of phenethanolamine compounds in tissue specimens. Such difficulties have been eliminated in the technique of this report permitting rapid and sensitive analysis of the phenethanolamine compounds regardless of the type of biologic specimen. The ability to rapidly remove the *n*-hexane under vacuum permits considerable concentration of the product which allows for a significant further increase in sensitivity.

Ephedrine and its immediate metabolite norephedrine are determined simultaneously, and in the data of Table IV are reported as total ephedrine compounds. In specimens from man this creates no difficulty because nearly all of the drug is excreted in an unchanged form (13, 15) with a small percentage appearing as norephedrine.

The high polarity of the hydroxyl group in phenethanolamine compounds necessitates the use of diethyl ether or an equivalent polar substance as the initial extracting solvent. The low affinity of ephedrine for fat depots (Table IV) also illustrates the *in vivo* polar properties of the drug and evidently accounts for its rapid renal excretion. The polar nature of these molecules is probably responsible for the higher blood levels observed in our laboratory for ephedrine when compared to blood levels for amphetamine and other related basic drugs after each were adminsitered to animals at equivalent dose levels (16). The enhanced sensitivity of the method proposed in this report now makes practical the UV spectrophotometric determination of ephedrine in blood specimens.

Interference by other drugs in the qualitative determination of the phenethanolamine structure is minimal. Phenyramidol can easily be identified, since its UV spectrum is very characteristic and quite different from that of the ergot alkaloids. Although the technique does not distinguish between certain other of the phenethanolamine compounds, this lack of distinction does not decrease the forensic toxicologic importance of the procedure.

Periodate is effective in the determination of ephedrine only if the oxidation takes place in an alkaline system. If spectra of both the benzaldehyde and its corresponding semicarbazone are utilized in the analysis, a procedure with excellent specificity and sensitivity is available.

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