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Colorimetric Determination of Catecholamines by 2,3,5-Triphenyltetrazolium Chloride

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Abstract □ A convenient spectrophotometric method was developed for the determination of epinephrine, levarterenol, isoproterenol, and methyldopa by reduction of 2,3,5-triphenyltetrazolium chloride and subsequent measurement of the formazan at 485 nm. With absolute alcohol as the solvent, maximum color absorption was attained in 30 min at 25° in the presence of 0.1 *N* KOH. Evidence is provided to account for the reduction of the tetrazolium salt at the expense of the epinephrine catechol moiety. In addition to the considerably high values of the molar absorptivities of the chromogen formed, ideal adherence of the color absorption to the Beer–Lambert law permitted a sensitive microdetermination of these catecholamines in both pure forms and pharmaceutical formulations. The tetrazolium interaction was selective. No interference was encountered from common catecholamine antioxidants, adjuvants, or noncatechol degradation products.

Keyphrases □ Epinephrine—colorimetric analysis in bulk drug and dosage forms □ Levarterenol—colorimetric analysis in bulk drug and dosage forms □ Isoproterenol—colorimetric analysis in bulk drug and dosage forms □ Methyldopa—colorimetric analysis in bulk drug and dosage forms □ Colorimetry—analyses, epinephrine, levarterenol, isoproterenol, and methyldopa in bulk drug and dosage forms □ Catecholamines, various—colorimetric analyses in bulk drug and dosage forms □ Adrenergic agents—epinephrine, levarterenol, and isoproterenol, colorimetric analyses in bulk drug and dosage forms □ Antihypertensives—methyldopa, colorimetric analysis in bulk drug and dosage forms

The presence of various catecholamine congeners in many pharmaceutical formulations necessitates a rapid, economical, and sensitive analytical method. Most of these derivatives are officially assayed by chemical, titrimetric, and spectrophotometric procedures (1, 2). Because of their phenolic and basic functions, catecholamines interact with many chromogenic reagents such as potassium ferricyanide–ferric chloride (3), alkali ferrocyanates (4), *p*-nitrophenyldiazonium chloride (5), nitrosomercurials (6), and alkali molybdates (7). Other earlier reported chromogens include iodine, ferric ion, nitrous acid, and 1,2-naphthoquinone 4-sodium sulfonate (8). However, accurate colorimetric determinations of pharmaceutical catecholamines using their reducing potential have not been developed.

The present study investigated 2,3,5-triphenyltetrazolium chloride as a convenient reagent for the colorimetric determination of epinephrine, levarterenol, isoproterenol, and methyldopa, both in pure forms and dosage formulations. The fact that tetrazolium salts can be reduced selectively into highly colored formazan dyes constituted the basis of the current work (9).

EXPERIMENTAL

Instrumentation—A double-beam spectrophotometer[1], a pH meter[2] fitted with a sealed calomel electrode, a shielded glass electrode, and a suitable thermostated[3] water bath were used.

Catecholamines—Pharmaceutical grade epinephrine hydrochloride, levarterenol bitartrate, isoproterenol hydrochloride, and methyldopa were utilized as the working standards.

Catecholamine Dosage Forms—The following commercial formulations were analyzed: methyldopa tablets[4], epinephrine injection[5], procaine–epinephrine injection[6], levarterenol injection[7], and isoproterenol solution[8].

Reagents—*Tetrazolium Solution*—Dissolve 0.5 g of pure 2,3,5-tri-

[1] Spektromom-203, MOM, Budapest, Hungary.
[2] Radelkis OP-401/2, Budapest, Hungary.
[3] T-606 MTA, Budapest, Hungary.
[4] Contains 250 mg of methyldopa/tablet; El-Kahira-MSD, Cairo, Egypt.
[5] Contains 1.0 mg of epinephrine/1-ml ampul; Misr Co. for Pharmaceuticals, Cairo, Egypt.
[6] Contains 10.0 μg of epinephrine and 10.0 mg of procaine hydrochloride/1-ml ampul; El-Nile Co. for Pharmaceuticals, Cairo, Egypt.
[7] Contains 1.0 mg of levarterenol/1-ml ampul; El-Nile Co. for Pharmaceuticals, Cairo, Egypt.
[8] Isoprenaline, contains 10.0 mg of isoproterenol hydrochloride/1 ml of solution; El-Nile Co. for Pharmaceuticals, Cairo, Egypt.

Table I—Effect of Potassium Hydroxide on Epinephrine–Tetrazolium Interaction

Milliliters Added per 10 ml of Assay Solution[a]		
0.1% KOH	0.5% Tetrazolium Chloride	Absorbance, 485 nm
0.50	3.0	0.050
0.50	5.0	0.080
0.50	7.0	0.120
1.00	3.0	0.290
1.00	4.0	0.310
1.00	5.0	0.370
1.00	6.0	0.370
2.00	3.0	0.080
2.00	5.0	0.080
2.00	7.0	0.105

[a] *Containing* 1.0 μg of epinephrine base/ml.

phenyltetrazolium chloride[9] in 100 ml of absolute ethanol[10]. This reagent should be freshly prepared, although it may be kept for longer periods if stored in a dark, cool place.

Potassium Hydroxide Solution—Prepare 0.1% (w/v) carbonate-free[9] solution in absolute ethanol.

Standard Solutions—Dissolve an accurately weighed amount of the appropriate working standard in absolute ethanol and dilute the solution quantitatively and stepwise with absolute ethanol to obtain a final concentration of 10.0 μg of catecholamine base/ml.

Assay Samples—*Injections and Solutions*—Pipet 1.0 ml, or the measured contents of a single-dose container of the injection, into a suitable volumetric flask and dilute with absolute ethanol to obtain ~10 μg of the claimed amine content/ml of the prepared solution.

Tablets—Place a single powdered tablet, or its equivalent from a composite of 20 tablets, in a 100-ml volumetric flask and add 5 ml of 1% (w/v) HCl and 20 ml of absolute ethanol. Allow the mixture to stand for 30 min with frequent shaking, bring to volume with absolute ethanol, mix well, and filter through a dry filter into a dry flask. Discard the first portions of the filtrate. Dilute an aliquot of this sample with absolute ethanol to afford a concentration of ~10 μg of the claimed amine content/ml of the prepared solution.

Procedure—Pipet 1.0 ml of the standard (or of the appropriately prepared sample) solution into a 10-ml volumetric flask. Add, in order, 5.0 ml of tetrazolium solution and 1.0 ml of potassium hydroxide solution; then dilute to volume with absolute ethanol, mixing well after each addition. Allow the solution to stand in the dark in a 25.0 ± 0.1° water bath for 30 min. Transfer the solution into a 1-cm glass cell and determine its absorbance at 485 nm *versus* a blank prepared from 1.0 ml of absolute ethanol and treated similarly.

RESULTS AND DISCUSSION

Tetrazolium–Epinephrine Interaction—The utility of 2,3,5-triphenyltetrazolium chloride as an efficient reagent for both detection and colorimetric determination of α-ketolic steroids (10, 11), reducing sugars (12, 13), and ascorbic acid (13, 14) is well documented. For ascorbic acid, use is made of the reducing potential of the α,β-diol groups for the transformation of the tetrazolium salt into a highly colored formazan derivative.

Catecholamines constitute an important class of pharmaceutical compounds that contain such dihydroxy groups within a phenolic structure and, therefore, may make the reduction of tetrazolium salts possible. Since this reduction is generally enhanced by the use of strong bases (9), considerable care was taken to guard against the instability of catecholamines in an alkaline medium.

Preliminary trials to induce the reduction of 2,3,5-triphenyltetrazolium chloride by ethanolic solutions of epinephrine hydrochloride in the presence of such weakly basic buffers as 1.0% (w/v) aqueous solutions of sodium acetate or sodium metaborate (pH ~8.0–8.5) were almost futile. However, an intense red-colored product, with maximum absorption at 485 nm, was readily formed when the interaction was carried out in the presence of ~0.05–0.25% (w/v) alcoholic potassium hydroxide solutions. At higher alkali concentrations, another weak absorption interfered at 395 nm and was more prominent when the interaction was effected in the presence of 0.30% (w/v) alcoholic tetraethylammonium hydroxide. The latter is usually used in much higher concentrations (~1.0%) as the conventional basic medium prescribed for the reduction of 2,3,5-triphenyltetrazolium chloride (8).

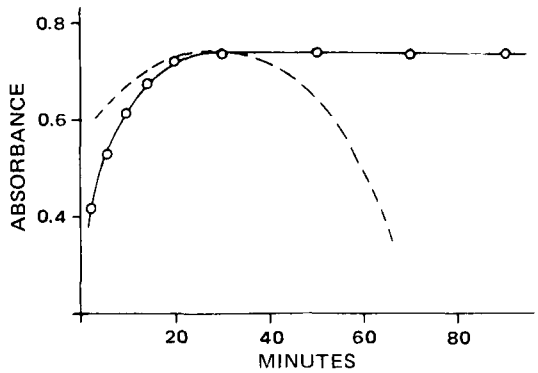


Figure 1—*Effect of time and temperature on color development at 25° (—) and at boiling water bath temperature (- - -).*

No interference with formazan color absorption at 485 nm could be traced when 1.0 ml of ~0.20–0.30% (w/v) ethanolic potassium hydroxide was added to 1.0 ml of the standard epinephrine hydrochloride solution that was already mixed with different volumes of the tetrazolium salt solution. Obviously, this order of addition reduced side reactions between epinephrine and the alkali.

A further rigorous investigation was carried out to assess the least alkali concentration that would induce maximum interaction between fixed aliquots of the tetrazolium reagent and standard epinephrine solutions. An appreciable increase of the 485-nm color absorption was achieved by use of 0.10 mg of potassium hydroxide and 2.50 mg of tetrazolium chloride/ml of the final catecholamine solution for assay (Table I). A concurrent investigation of the color–time curve (Fig. 1) indicated a 30-min interaction period to bring color development to a constant at 25°. The formazan remained stable for at least 24 hr.

Reduction of the tetrazolium reagent was accelerated by carrying out the interaction with epinephrine at the temperature of a boiling water bath. However, a gradual destruction of the intense red color followed, with a distinct drop in the absorptivity after ~40 min (Fig. 1). Moreover, no reproducible measurements of the color absorption could be attained. This result might be a consequence of competitive side oxidation reactions of the catecholamine at such elevated temperature. Potential interference with the tetrazolium–epinephrine interaction because of fluctuations in the reaction temperature was eliminated by carrying out the process at 25°.

Reaction Mechanism—A strong reducing group is a decisive criterion for an efficient interaction with 2,3,5-triphenyltetrazolium chloride, whose oxidation–reduction potential approximates −0.2 v (9). Such a condition can be fulfilled by the amine and/or catechol moieties of epinephrine, each of which is known to be oxidized selectively (15). Clarification of this point was achieved by an investigation of 2,3,5-triphenyltetrazolium chloride interactions with catechol, ephedrine sulfate, and phenylephrine hydrochloride.

Neither ephedrine nor phenylephrine could induce the formation of formazan under various experimental conditions, while a direct red color response was manifested by catechol, with a molar absorptivity of 4.14×10^4 at 485 nm. This finding indicated a key effect for the catechol

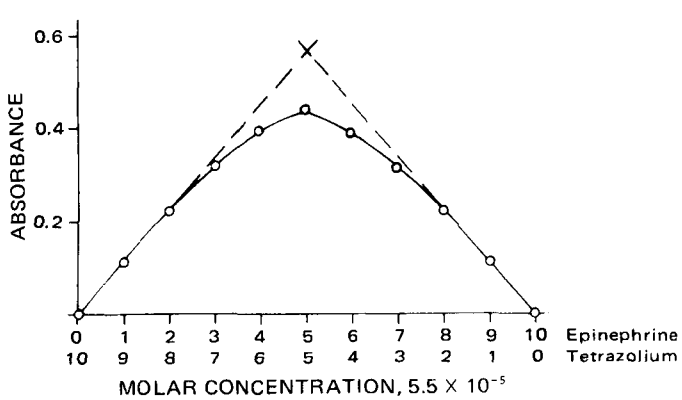


Figure 2—*Molar variation curve of the epinephrine–tetrazolium interaction at 25° in the presence of 1.0 ml of 0.1% KOH solution.*

[9] BDH, England.
[10] Aldehyde-free spectrograde, Merck, Germany.

Table II—Analysis of Epinephrine Standard Solutions

Replication	Epinephrine, Added, μg/ml	Found, %[a]	SD
1	0.25	100.37	0.66
2	0.50	99.59	0.75
3	0.75	99.68	0.57
4	1.00	100.50	0.61
5	1.50	100.28	0.58
6	2.00	99.70	0.45
7	2.50	100.27	0.56
8	3.00	99.65	0.62
	Mean recovery, % = 100.00		
	Pooled SD, $\pm S_p$, % = 0.38		

[a] Average of five determinations.

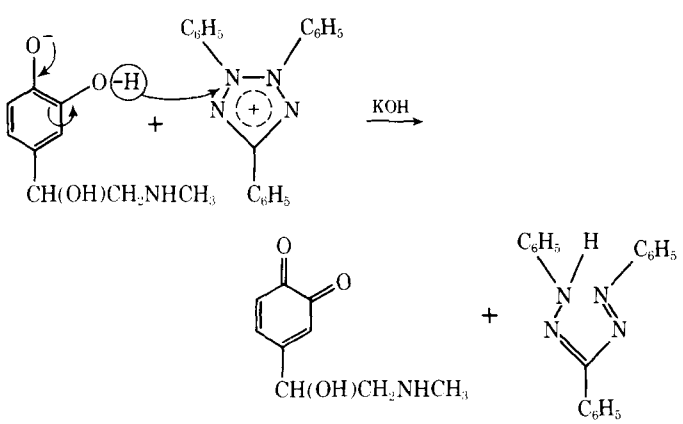
function in bringing about the oxidation of epinephrine by the tetrazolium salt. In fact, this reaction can be considered as an extension of the reduction of epinephrine by aliphatic diols (12–14) into the aromatic series.

As assessed by the continuous molar variation method (Fig. 2), epinephrine interacts with 2,3,5-tetrazolium chloride on a unimolar basis. Only limited suggestions can be made concerning the nature of the oxidation product(s) of the catecholamine. Mainly, formation of *o*-quinone derivatives would be expected since similar products are quite common for catechol and epinephrine (15, 16). Supporting this possibility is the nature of tetrazolium salts as efficient hydride-ion acceptors (17–19). Since the catechol-derived phenolate anion has hydride-ion donor properties (20), an interpretation of the entire tetrazolium–epinephrine interaction in terms of an intermolecular hydride-ion transfer reaction (Scheme I) seems plausible.

Quantitative Analysis—At fixed experimental conditions, the quantity of the formazan depended on the amount of epinephrine added to reduce the tetrazolium salt. A linear regression analysis of the Beer's plot at 485 nm revealed excellent adherence (r = 0.9996) up to a concentration of 3.0 μg (A = ~1.1) of the catecholamine/ml of the assayed solution. This result permitted the development of the interaction into a sensitive spectrophotometric analysis of epinephrine on account of the high value of the molar absorptivity observed, $\epsilon_a = 6.78 \times 10^4$.

Other catecholamines that behaved similarly with 2,3,5-tetrazolium chloride included working standards of levarterenol, isoproterenol, and methyldopa, with ϵ_a values corresponding to 3.38×10^4, 5.48×10^4, and 6.61×10^4, respectively (calculated with respect to the molar concentration of the free anhydrous bases). Replicate analysis of epinephrine working standards (Table II) revealed both high accuracy and sensitivity of the investigated colorimetric determination.

Assay of Dosage Forms—2,3,5-Triphenyltetrazolium chloride was not reduced by chlorobutanol, sodium metabisulfite, procaine hydrochloride, acacia, or starch. These ingredients constitute the major antioxidants, common adjuvants, and tablet excipients encountered in many catecholamine dosage forms. Potential interference by reducing sugars could be eliminated by alcohol extraction prior to analysis. As revealed by the data given in Table III, the tetrazolium method proved applicable to the analysis of different dosage forms of the studied catecholamine derivatives with remarkable accuracy.



Scheme I

Table III—Analysis of Dosage Forms of Epinephrine, Levarterenol, Isoproterenol, and Methyldopa

Preparation[a]	Catecholamine Content, mg/Unit Label Claim	Found[b]	Added	Recovered
Epinephrine injection	1.0	0.98	1.0	1.97
Procaine–epinephrine injection	0.010	0.011	0.020	0.031
Levarterenol injection	1.0	0.97	0.50	1.45
Isoproterenol solution	10.0	9.86	5.0	14.90
Methyldopa tablets	250	247.3	100	346.6

[a] See *Experimental* for additional details. [b] Average of three determinations.

Interference with catecholamine analysis by noncatechol degradation products such as adrenochrome and adrenoquinone was not observed. Since these products are the major degradation derivatives of most catecholamines and are usually encountered in cases of autoxidation and metal-catalyzed and aerobic oxidations (21), this result indicates that the presented method is stability indicating in cases where the decomposition products do not have intact catechol moieties. Apart from its general validity for the analysis of catecholamines, the proposed procedure offers several advantages over current methods in terms of time, sensitivity, and convenience. It should also have utility in automated assays.

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