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Analysis of oxidation products of 10-[3′-N-benzylaminopropyl]-phenoxazine redox indicator by spectral and cyclic voltammetric methods

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Abstract. Synthesis of 10-[(3'-N-benzylamino)propyl]phenoxazine [BAPP] is accomplished in two steps. The first step involved N^{10}-alkylation of parent phenoxazine via phase transfer catalysis (PTC) followed by iodide-catalysed nucleophilic substitution of the N^{10}-propyl chloride with N-benzylamine. BAPP formed is purified by column chromatography. In the second stage BAPP undergoes one-electron oxidation with Ce(IV) to form a pink radical cation [$BAPP^{+\bullet}$]. In the presence of more than one equivalent of Ce(IV), the radical cation undergoes a second one-electron oxidation to form a brownish yellow coloured dication [$BAPP^{2+}$], which is characterized by UV-Vis, IR and mass-spectral methods. The biological functions of phenoxazines are connected to a great extent with their ability to undergo reversible redox conversion and therefore the electrochemical behaviour of BAPP is investigated by cyclic voltammetry. Other cyclic voltammetric parameters have also been determined. Bromine in acid medium oxidizes BAPP to three products as shown by HPLC. The tentatively predicted structures based on the mass-spectral data support the formation of three brominated oxidized products. In order to explore the analytical applications, the optimum conditions for the use of BAPP as redox indicator in the macro and micro estimation of ascorbic acid, methionine, isoniazid, phenylhydrazine hydrochloride and biotin using chloramine-T as an oxidant have been developed. The indicator gives sharp and stoichiometric end-points. During the titration, BAPP initially undergoes a reversible one-electron oxidation. With progress of titration, the radical pink cation is oxidized to a blue coloured dication with the loss of one more electron. The utilization of BAPP as an indicator for oxidation–reduction reactions for the volumetric determination of bioanalytically important species such as ascorbic acid, methionine and isoniazid in real samples is significant.

Keywords. Phenoxazine oxidation; cyclic voltammetry; redox indicator.

1. Introduction

Compounds of pharmacological interest have been found among phenoxazine derivatives and they have been claimed to be nervous system depressants in particular with sedative, antiepileptic, tranquillizing, spasmolytic, antitubercular and antihelminthic activities. Recently, Thimmaiah *et al*[1–4] have prepared a number of phenoxazine derivatives and

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examined their ability to reverse the resistance of the cancer cells. Analogous to phenothiazines[5], the pharmacological activity of phenoxazines could be due to their metabolites. For example, a phenothiazine cationic radical, an oxidized species, is generally assumed to be a metabolic intermediate in the formation of sulphoxide and hydroxylated products *in vivo*[6] and it has been demonstrated that these metabolites can be formed from cation radical reaction in aqueous buffer[7]. It is also speculated that analogous to phenothiazines, phenoxazines could also undergo metabolism *in vivo* to form the intermediates via oxidized species such as radical cations and dications as intermediates.

Phenoxazine derivatives are electron donors that readily form radical cations. The change in colour from colourless to pink due to the formation of radical cations and then to brownish yellow due to the formation of dications can be attributed to the 2-electron oxidation of phenoxazines. In view of the formation of the radical cations and dications from phenoxazines during oxidation and also *in vivo* as metabolic intermediates; the understanding of their mechanism of oxidation is of paramount importance. Therefore, the authors have selected 10-[(3′-N-benzylamino)propyl]phenoxazine [BAPP] and studied the mechanism of oxidation of this compound by spectral and cyclic voltammetric methods. Further, in order to find its application in analytical chemistry, the authors have proposed this reagent as a sensitive redox indicator in the titrimetric estimation of biologically important compounds.

2. Experimental

2.1 *Apparatus*

UV-visible spectra were recorded in methanol using Jasco model 610 spectrophotometer with 1 cm matched silica cells. The infrared spectra of BAPP as KBr pellets and its oxidized products in nujol in the range 4000–400 cm^{-1} were recorded on a Perkin-Elmer model 1320 spectrophotometer. ^{1}H-(200 MHz) and ^{13}C-(50 MHz) spectra of 10-(3′-chloropropyl)phenoxazine were recorded in $CDCl_3$ solution in a 5 mm tube on an IBM NR 200 AF Fourier transform spectrometer and the ^{1}H-(60 MHz) spectrum of BAPP was recorded in $CDCl_3$ solution in a 5 mm tube on a Hitachi FT NMR model R-600 spectrometer with TMS as internal standard. The mass-spectral data were acquired using an Autospec Q (VG Analytical Manchester, UK) hybrid tandem mass spectrometer of $E_1BE_2 - qQ$ geometry (where E is an electric sector; and Q, a quadrupole mass analyser).

2.2 *Materials*

All the general chemicals and supplies were obtained from standard commercial sources unless otherwise indicated. Phenoxazine, 1-bromo-3-chloropropane, N-benzylamine, tetrabutylammonium bromide and biotin were purchased from Aldrich Chemical Co. (Milwaukee, Wisconsin, USA). Ascorbic acid, isonicotinic acid hydrazide, methionine and phenylhydrazine hydrochloride were purchased from SRL Pvt. Ltd., Bombay (India).

2.3 *Solutions*

Stock solutions of chloramine-T, ascorbic acid, methionine, isonicotinic acid hydrazide, phenylhydrazine hydrochloride and biotin were prepared and standardized by

recommended methods. A 0·1% solution of BAPP in ethanol/water was prepared and stored in an amber bottle.

2.4 *Procedure*

2.4a *Synthesis of 10-(3′-chloropropyl)phenoxazine:* 7·0 g of phenoxazine (0·04 mol) was dissolved in 40 ml of benzene and to this solution was added 175 ml of 6 N potassium hydroxide and 6·44 g of tetrabutylammonium bromide (0·02 mol). The reaction mixture was then stirred at room temperature for 60 min. 1-Bromo-3-chloropropane (0·1 mol) was slowly added to the reaction mixture and the mass stirred at room temperature for 24 h. Benzene was evaporated and the aqueous layer extracted with ether. The ether layer was washed with water and the organic layer was dried over anhydrous sodium sulphate and rotavaporated. The residue was chromatographed on silica gel. Petroleum ether-ethyl acetate (3:1) eluted pure 10-(3′-chloropropyl)phenoxazine as white crystals (7·94 g, 80%, m.p. 53°C). The purified compound was characterized by spectral methods.

2.4b *10-[(3′-N-benzylamino)propyl]phenoxazine:* To the solution of 1·5 g (5·77 mmol) of 10-(3′-chloropropyl) phenoxazine in 150 ml of anhydrous acetonitrile, 2·0 g of KI, 2·1 g of K_2CO_3 and 2·47 g (6·74 mmol, 2·52 ml) of N-benzylamine were added and refluxed overnight. After the completion of the reaction, as evidenced by TLC, the reaction mixture was diluted with water and extracted with ether (3 × 100 ml). The ether layer was washed with water and dried over anhydrous sodium sulphate and rotavaporated. The product was purified by column chromatography to give pure 10-[(3′-N-benzylamino)propyl]phenoxazine (1·02 g, 80%, m.p. 158°C). UV λ_{max} (ε) (MeOH) 204 (85829), 241 (66588) and 306 (22394) nm; IR 3255, 2940, 2850, 1585, 1460, 1435, 1410, 1270, 1130, and 730 cm^{-1}; ^1H NMR (δ) 7·2–7·7 (*m*, Ar-H, 13H), 3·8–4·1 (*m*, H_k, H_m, H_n), 2·05 (*m*, 2H, H_l); EIMS (*m/z*) 331$[M + H]^+$.

2.4c *UV-visible spectra of oxidation products of BAPP:* A freshly prepared 10^{-4} M solution of BAPP was treated with 0, 0·25, 0·5, 1·0, 1·5, 2·0 or 3·0 equivalents of cerium(IV) sulphate in 0·5 M sulphuric acid and the absorption spectra were recorded at room temperature in the range 200–600 nm.

2.4d *Cyclic voltammetry:* A 50 ml solution of 6×10^{-4} M phenoxazine or BAPP in anhydrous acetonitrile containing 0·1 M with respect to tetrabutylammonium perchlorate was prepared and deoxygenated by bubbling with dry nitrogen gas for 15 min prior to all the runs. The electrode potential was scanned between + 100 and + 1200 mV at a scan rate of 12, 24, and 48 mV per second at room temperature in a one compartment cell consisting of microplatinum as working electrode, large platinum foil as counter electrode and saturated calomel electrode connected via luggin capillary as reference electrode.

2.4e *Titration of 0·05–0·005 N ascorbic acid and phenylhydrazine hydrochloride:* 20 ml of 0·05–0·01 N ascorbic acid or phenylhydrazine hydrochloride, 4 ml of 10% potassium bromide and 0·2 ml of 0·1% BAPP or 10 ml of 0·01–0·005 N ascorbic acid, 2 ml of 10% potassium bromide and 0·1 ml of 0·1% BAPP were all mixed and diluted to 40 ml or 25 ml respectively with enough sulphuric, hydrochloric, phosphoric or acetic acid and titrated with 0·05–0·01 N CAT and 0·01–0·005 N CAT solutions respectively until the appearance of a blue colour.

2.4f *Titration of 0·05–0·005 N methionine, isonicotinic acid hydrazide or 0·01–0·0025 N biotin:* 20 ml of 0·05–0·01 N methionine or isonicotinic acid hydrazide solution, 4 ml of 10% potassium bromide or 10 ml of 0·01–0·005 N methionine, isonicotinic acid hydrazide or 0·01–0·0025 N biotin and 2 ml of 10% potassium bromide were mixed and diluted to 40 ml or 25 ml with enough sulphuric, hydrochloric or phosphoric acid and titrated with 0·05–0·01, 0·01–0·005 or 0·01–0·0025 N CAT solution until the appearance of blue colour adding 0·2 ml or 0·1 ml of 0·1% BAPP indicator near the end-point.

2.4g *Procedure for the assay of ascorbic acid and isoniazid in pharmaceutical preparations:* An accurately weighed amount of well powdered tablets containing vitamin C in the range 75–500 mg or 100–300 mg of INH was stirred in doubly distilled water for about 15 min. The residue was filtered through Whatman No. 42 filter paper and washed with water. The filtrate was made up to 100 ml and different aliquots of this solution were titrated following the recommended procedure and the amount of vitamin C or INH was calculated (in INH titration the indicator was added near the end-point).

2.4h *Determination of ascorbic acid in citrus fruits:* Fresh, yellow lemon, orange or red tomatoes were taken and juice extracted as quickly as possible to prevent aerial oxidation of ascorbic acid before analysis. Then, the juice was filtered through Whatman No. 42 filter paper and diluted with doubly distilled water to 100 ml. Different aliquots were titrated following the recommended procedure and ascorbic acid content calculated.

2.4i *Determination of methionine in aminodrip:* A known volume of aminodrip solution was transferred into a 100 ml standard flask and made up to the mark. Different aliquots of this solution were titrated following the recommended procedure and the amount of methionine content calculated.

3. Results and discussion

3.1 *N-Alkylation of phenoxazine via phase transfer catalysis*

Phenoxazine has a less basic nitrogen atom and the previously described preparative procedure for N-alkylation of this compound[2] needed sodamide in liquid ammonia. However, this compound undergoes N-alkylation in the presence of phase transfer catalyst (PTC) more easily compared to the previously described preparative procedures. Stirring of parent phenoxazine at room temperature with 1-bromo-3-chloropropane in a two phase system consisting of an organic solvent (benzene) and 6 N aqueous potassium hydroxide solution in the presence of tetrabutylammonium bromide [$(n\text{-}C_4H_9)_4N^+Br^-$] leads to the formation of 10-(3′-chloropropyl)phenoxazine in good yield. Here, ammonium salt transports hydroxide ion from the aqueous phase to the organic phase where the actual reaction takes place. These results are interpreted by deprotonation of phenoxazine by [OH^-], transferred by the catalyst into the organic layer. The anion formed may be regarded as phenolate stabilised anion, which subsequently undergoes alkylation to form the aromatized system. 10-[(3′-N-benzylamino)propyl]phenoxazine (BAPP) was prepared by iodide catalyzed nucleophilic substitution of the N^{10}-propyl chloride with N-benzylamine. The product was purified by column chromatography, dried under high vacuum and characterized by UV-, IR-, ^1H NMR and mass spectral studies. The UV-spectrum of BAPP showed three λ_{max} values at 204, 241 and 306 nm, which may be

assigned to $\pi \rightarrow \pi^*$, $\pi \rightarrow \pi^*$ and $n \rightarrow \pi^*$ transitions. The IR band at 3255 cm^{-1} region may be assigned to the C–H stretching frequency. The ^{1}H-NMR spectrum showed thirteen aromatic protons and the data are in accordance with the structure assigned. The assignment of protons is fully supported by the integration curves. The mass spectrum showed an intense molecular ion [M^+] peak at *m/z* 331. The spectral data are consistent with the assigned structure.

3.2 *Characterization of the oxidized products of BAPP by spectral methods*

The absorption spectrum of BAPP after oxidation by cerium(IV) was recorded and the λ_{max} and molar extinction coefficient (ε) values in the visible region are given in table 1. Visible spectrum in the presence of stoichiometric amounts of BAPP and Ce(IV) [1: 0·25 (A), 1: 0·50 (B), 1: 1·00 (C)] in figure 1a and the stoichiometric amounts of BAPP and Ce(IV) [1: 1·50(A), 1: 2·00 (B), 1: 3·00 (C)] in figure 1b are given. BAPP undergoes a reversible one electron oxidation to form a radical cation [$BAPP^{+\bullet}$] which is characterized by two λ_{max} values at 415 nm and 532 nm in the visible region. The intensity of the pink colour due to the formation of radical cation [$BAPP^{+\bullet}$] at λ_{max} 532 nm reached the maximum at the stoichiometric amount [BAPP:Ce(IV) = 1:1] of the oxidant as evidenced by a substantial increase in molar extinction coefficient value, that is, from 3830 to 7360 dm^{-3} mol^{-1} cm^{-1} (table 1). In order to examine the fate of the radical cation, concentration of cerium(IV) was increased [BAPP:Ce(IV) = 1:1·5, 1:2 and 1:3] further. In the presence of more than one equivalent of cerium(IV), radical cation underwent a second one-electron oxidation to form a dication [$BAPP^{2+}$] (scheme 1). Examination of the spectrum revealed that the oxidation of [$BAPP^{+\bullet}$] to [$BAPP^{2+}$] resulted in a drastic change in the intensity of the peaks at λ_{max} 532 and 415 nm. The ε value of the dication [$BAPP^{2+}$] at 415 nm increased from 590 to 2580 dm^{-3} mol^{-1} cm^{-1} and at 532 nm reduced from 7360 to 1220 dm^{-3} mol^{-1} cm^{-1}. The drastic reduction of the 'ε' value at 532 nm with increasing concentration of cerium(IV) suggested that the pink-coloured cation radical [$BAPP^{+\bullet}$] was further oxidized to a brownish yellow coloured dication [$BAPP^{2+}$]. However, at 1:3 stoichiometric ratio the peak at 532 nm due to radical cation completely disappeared indicating that the radical cation was completely oxidized to a dication. It was of interest to note that stoichiometric amounts [BAPP:Ce(IV) = 1:1] resulted in the quantitative first one-electron oxidation of neutral BAPP to form the radical cation [$BAPP^{+\bullet}$] (scheme 1). Radical cation and dication species were also obtained when BAPP was oxidized by hydrogen peroxide in the presence of 1 M sulphuric acid. Although two equivalents of cerium(IV) [BAPP:Ce(IV) = 1:2] were required theoretically for the

Table 1. Visible spectral data for the oxidation products of BAPP.

Number of equivalents of Ce(IV)*	$\lambda_{max\,(nm)}$ (ε, dm^{-3} $mol^{-1}cm^{-1}$)	$\lambda_{max\,(nm)}$ (ε, $mol^{-1}cm^{-1}$)
0·25	532 (3830)	415 (590)
0·50	532 (5990)	415 (1630)
1·00	532 (7360)	415 (2030)
1·50	532 (2990)	415 (2100)
2·00	532 (1220)	415 (2580)
3·00	Disappears	–

*The numbers are all ratios against BAPP taken as one unit

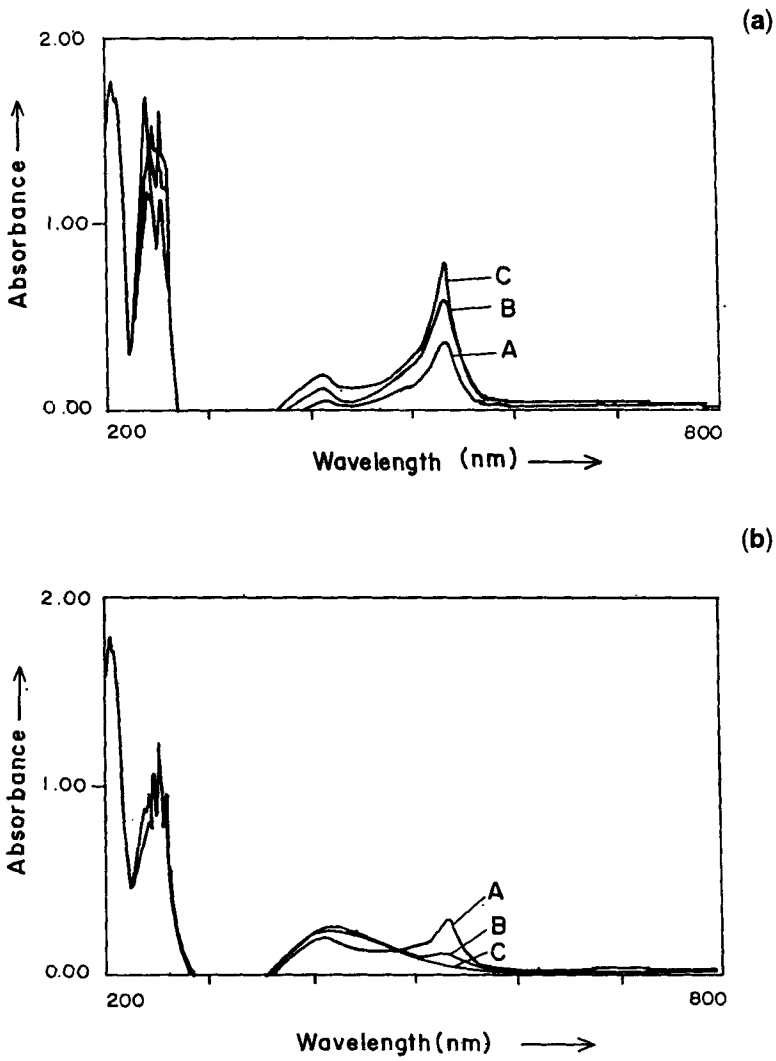
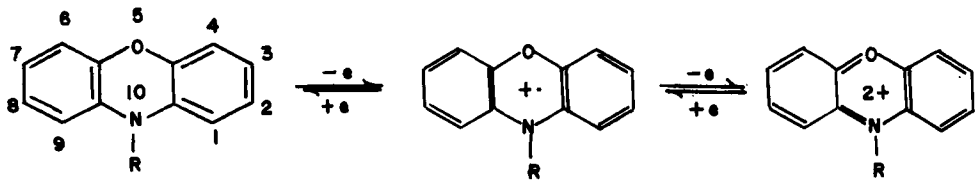


Figure 1. Visible spectrum in the presence of stoichiometric amounts of (a) BAPP:Ce(IV) [1:0·25(A), 1:0·50(B), 1:1·00(C)] and (b) BAPP:Ce(IV) [1:1·50(A), 1:2·00(B), 1:3·00(C)].

quantitative two electron-oxidation of BAPP to $BAPP^{2+}$, in actuality, three equivalents of cerium(IV) were involved suggesting that the oxidation of radical cation to dication is kinetically a slow process[8] The IR signals at 3245, 2950, 2845, 1590, 1465, 1435, 1400, 1265, 1140 and 740 cm^{-1} indicated the presence of characteristic functional groups of the phenoxazine type of molecule. The mass spectrum of dication was recorded and it displayed the protonated dication peak at *m/z* 331 (figure 2). Phenoxazines being weak bases, they usually get protonated in mass-spectral analysis. Examination of the mass spectral data revealed that the oxidized products of BAPP yield abundant molecular ion either in the monoprotonated or diprotonated form. The molecular ion peak is the base peak. The phenoxazine ring system remains stable, whereas fragmentation reactions were·



POZ, R = -H

BAPP, R = $-CH_2-CH_2-CH_2-NH-CH_2-$ (k) (l) (m) (n)

Scheme 1. Proposed mechanism of oxidation of phenoxazine or 10-[3′-N-benzylaminopropyl]phenoxazine.

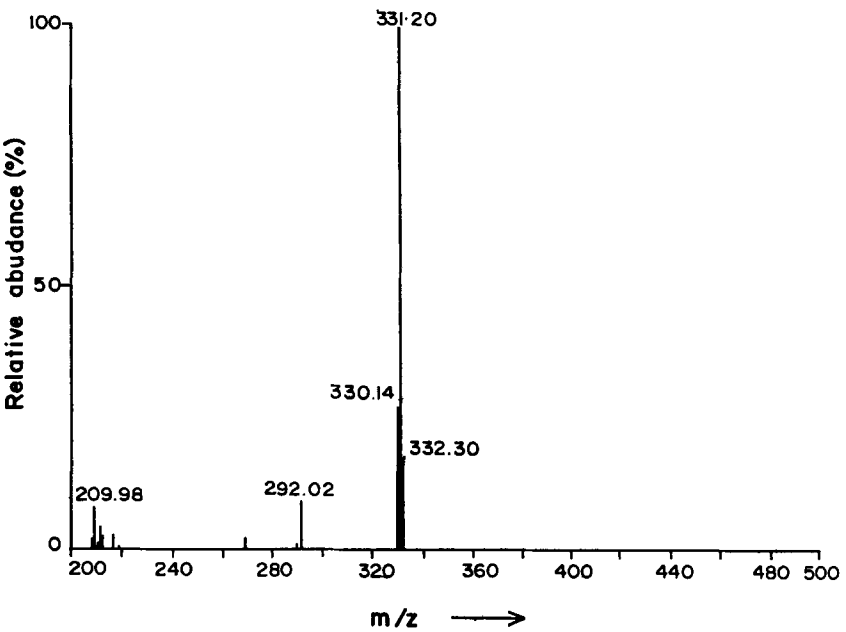


Figure 2. Mass spectrum of oxidized product of 10-[3′-N-benzylaminopropyl]-phenoxazine.

observed due to cleavage of bonds in the N^{10}-side chain portion. Since the molecular weight of the dication remained unchanged even after consuming more than one equivalent of Ce(IV), it can be easy to deduce that BAPP has lost only two electrons to form the dication.

3.3 *Electrochemical oxidation of BAPP*

Table 2 lists the cyclic voltammetric parameters for POZ and BAPP. The cyclic voltammogram of BAPP has the appearance as shown in figure 3b and it exhibited two reversible anodic waves at 650 mV and 956 mV and two cathodic waves at 585 mV and 844 mV at a scan rate of 28 mV/s. The peak at 650 mV corresponds to the oxidation of the neutral molecule to the radical cation [$BAPP^{+\bullet}$] and the second anodic peak of 956 mV stands for the oxidation of radical cation to dication [$BAPP^{2+}$]. The first and the second redox potentials of BAPP were found to be 617 mV and 900 mV respectively. Of particular note was that the second cathodic peak (figure 3b) was found to be not significant suggesting that the dication is highly reactive.

4. BAPP as a redox indicator in titrations with chloramine-T

A survey of literature has revealed that, only very few ring substituted phenoxazines have been used as indicators in the titrimetric determination of various reductants[9–14]. Most of these methods suffer from one or more limitations. For example, some of the reported methods have revealed that the titrations were carried out at higher temperature. Further, it is also noted in the literature that no N^{10}-substituted phenoxazines have been proposed so far as redox indicators. The analytical aspects of N^{10}-substituted phenoxazines as redox indicators is of great importance because some of the 2,10-disubstituted phenoxazines have suitable redox potentials. Therefore, the authors have proposed BAPP as a sensitive redox indicator in the titrations of ascorbic acid, methionine, isonicotinic acid hydrazide, phenylhydrazine hydrochloride and biotin with CAT.

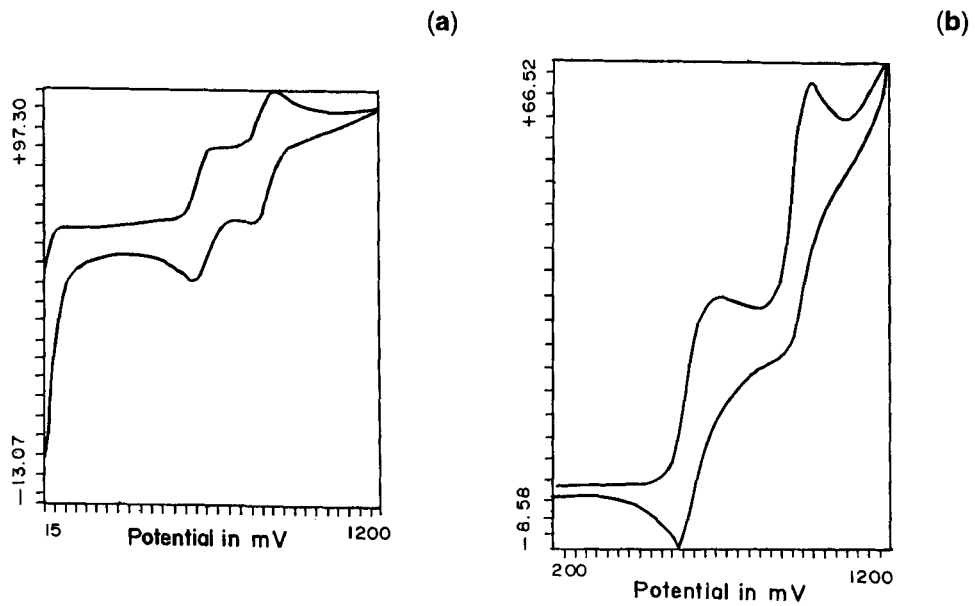


Figure 3. Cyclic voltammogram of (**a**) parent phenoxazine and (**b**) 10-[3′-N-benzylaminopropyl]phenoxazine.

Table 2. Cyclic voltammetric parameters of phenoxazine (POZ) and 10-[3′-N-benzylaminopropyl]phenoxazine (BAPP).

Compound	Scan rate (mV/s)	E_p^{01} (mV)	E_p^{r1} (mV)	E_{f1} (mV)	ΔE_{p1} (mV)	E_p^{02} (mV)	E_p^{r2} (mV)	E_{f2} (mV)	ΔE_{p2} (mV)	i_p^{01} (μA)	i_p^{r1} (μA)	$\frac{i_p^{r1}}{i_p^{01}}$	i_p^{02} (μA)	i_p^{r2} (μA)	$\frac{i_p^{r2}}{i_p^{02}}$
POZ	12	611	527	569	84	823	746	785	77	24	12	0·5	37	3.0	0·08
	24	605	537	571	68	825	750	788	75	21	10	0·47	37	3.2	0·08
BAPP	12	669	593	631	76	962	882	922	80	30	17	0·56	61	18	0·29
	24	650	585	617	65	956	884	900	112	32	20	0·62	62	19	0·4

E_p^{01} and E_p^{02}: anodic peak potentials, E_p^{r1} and E_p^{r2}: cathodic peak potentials; E_p^{f1} and E_p^{f2}: formal redox potentials

i_p^{01} and i_p^{02}: anodic peak currents, i_p^{r1} and i_p^{r2}: cathodic peak currents; $D_1^{1/2}$ and $D_2^{1/2}$: diffusion coefficients

$\Delta E_{p1} = E_p^{01} - E_p^{r1}$ and $\Delta E_{p2} = E_p^{02} - E_p^{r2}$: difference between the anodic and cathodic peak potentials for the first and second e^- transfer respectively

4.1 *Oxidation of BAPP by chloramine-T method*

CAT oxidizes potassium bromide to bromine in acid medium and the liberated bromine oxidizes BAPP to three products. During oxidation, the colour changes from colourless to pink and then to blue. The mass spectral data of the products after separation by HPLC were as follows: band at *m/z* 564 was assigned to the 3,7,9-tribromo derivative of [$BAPP^{2+}$], signal at *m/z* 484 in the mass-spectrum was assigned to the formation of 3,7-dibromo derivative of [$BAPP^{2+}$] and signal at *m/z* 278 was assigned to 7-bromophenoxazone. Similar oxidation products were reported earlier by Kehrman and Musso[15], where phenoxazine (POZ) in benzene was oxidized by bromine. Careful examination of the mass-spectral data of the oxidized products of POZ or BAPP has created a great deal of interest. The bromine liberated by the oxidation of potassium bromide by CAT under acidic conditions seems to have promoted the phenoxazine nucleus to undergo electrophilic substitution reaction in positions 3, 7 and 9 along with 2-electron oxidation at the heterocyclic ring system. The predicted structures based on the mass-spectral data were in accordance with the published data[15]. Under these conditions, the compound is believed to undergo electrophilic substitution reaction with bromine, followed by oxidation to produce brominated dication. The stability of the blue coloured dication was examined and it was found to be stable for 2 h. Confirmation of the structure of these oxidized products requires additional spectral data. However, experiments are underway to separate the individual oxidized products of POZ and BAPP by HPLC and to characterize them by spectral methods.

4.2 *Determination of formal redox potentials and transition potentials of BAPP*

Schilt's[16] method was used and the respective first and second formal potentials of BAPP were found to be 654 mV and 782 mV. The formal potential values determined by Schilt's method were compared with those of the corresponding potential values determined by cyclic voltammetry. Further, for assessing the merit of a redox indicator,

Table 3. Typical results for the substances titrated in the presence of BAPP as redox indicator in sulphuric acid medium.

Reductant	Taken (mg)	Found* (mg)	Relative error (%)	Standard deviation (mg)	Reference method** (mg)
Ascorbic acid	88·16	88·75	−0·66	0·056	88·54 (0·03)
	1·77	1·80	−1·69	0·040	1·79 (0·05)
Methionine	75·12	75·69	−0·75	0·042	75·60 (0·03)
	2·97	3·02	−1·68	0·054	3·04 (0·03)
Isonicotinic acid hydrazide	34·09	34·55	−1·34	0·045	34·6 (0·04)
	1·41	1·44	−2·12	0·037	1·42 (0·02)
Phenyl hydrazine hydrochloride	36·26	36·96	+0·82	0·068	36·78 (0·06)
	1·77	1·79	−1·12	0·054	1·79 (0·04)
Biotin	12·60	12·78	−1·42	0·060	12·90 (0·05)#
	2·24	2·27	−1·34	0·022	2·10 (0·08)

*Average of 5 determinations, similar results were obtained in HCl and H_3PO_4; **Average of 5 determinations, standard deviation is given in parentheses; #Potentiometric method

the transition potential is very helpful. Therefore, the transition potential of BAPP in the titration of ascorbic acid with CAT was determined and the value found to be 757 mV.

4.3 *Titration of ascorbic acid*

CAT liberates bromine from potassium bromide in an acid medium which oxidizes ascorbic acid to dehydroascorbic acid. In the titration of 0·05–0·005 N ascorbic acid, BAPP gives sharp and reversible end-points with a sharp colour change from colourless to blue in 0·4–2·0 M H_2SO_4, 0·5–2·0 M HCl, 1·5–3·5 M H_3PO_4 or 1·5–3·8 M HOAc. The end-point colour is stable for 15 min. Premature end-points at higher acidities and sluggish end-points for the titrations of 0·01–0·005 N ascorbic acid in acetic acid medium were obtained.

BAPP has advantages over phenothiazine indicators in that it gives sharper end-points, more accurate titre values and has less indicator correction. Further, the amount of BAPP required for the indicator action is minimal (0·2 ml of 0·1%) compared to phenothiazines (2 ml of 0·1%).

Before the proposed method was applied to the determination of ascorbic acid in real samples, the effect of number of substances commonly found in pharmaceuticals was first assessed. The following amounts of tablet diluents and excipients do not interfere in the determination of 50 mg of ascorbic acid: starch (350 mg), gelatin (200 mg), talc (250 mg), stearic acid (250 mg), alginic acid (150 mg), citric acid (700 mg), oxalic acid (600 mg), tartaric acid (350 mg), sucrose (600 mg), dextrose (650 mg), reserpine (250 mg) and pulvisacacia (250 mg).

The titration with CAT is useful for the determination of ascorbic acid in vitamin-C tablets [Celin (Glaxo), Sukcee (IDPL), Chewcee (Lederle), Cobadex (Glaxo), Becosules (Omni-Protech), Becelac (Pfmix), Polybion (Merck), Becozym C Forte (Roche)] and results for the determination of ascorbic acid in vitamin C tablets were compared with those found by the *o*-dianisidine method[17] and the official method of British Pharmacopoeia and also agreed well with the claimed values on the labels in all the tablets (table 4).

Ascorbic acid present in citrus fruit juices has been determined and their results given in table 4 are comparable favourably with those obtained by the published method[18].

4.4 *Titration of methionine*

Bromine oxidizes sulphide group of methionine to sulphoxide. In the titrations of 0·05–0·005 N methionine, BAPP gives sharp and irreversible end-points. During titration, there is a colour change of the indicator from colourless to blue via pink in 0·4–1·5 M sulphuric, 0·2–1·8 M hydrochloric or 1·5–3·5 M phosphoric acid solution containing potassium bromide. The end-point colour is stable for 15 min. BAPP gives overstepping end-points at lower acidities and premature end-points at higher acidities. Sluggish end-points were obtained in acetic acid medium.

BAPP has advantages over indigocarmine in that it (i) gives sharper end-points and more accurate values, (ii) they have less indicator correction, and (iii) functions in three acid media, while indigocarmine functions only in acetic acid medium.

Table 4. Determination of bioanalytically important compounds in real samples using BAPP as a redox indicator.

Sample	Compound	British Pharmacopoeia's or Manufacturer's specification (mg)	Present method (mg)*			Reference method (mg)
			H_2SO_4	HCl	H_3PO_4	
Vitamin C tablets						
Celin (Glaxo)	Ascorbic acid	500	497·80 (0·040)	498·10 (0·054)	497·90 (0·027)	498·90 (0·022)
Becelac (Pfmix)	Ascorbic acid	75	74·30 (0·020)	74·50 (0·020)	74·30 (0·027)	74·60 (0·030)
Polybion (Merck)	Ascorbic acid	150	148·00 (0·054)	147·80 (0·034)	148·20 (0·032)	148·20 (0·040)
Suckcee (IDPL)	Ascorbic acid	500	501·10 (0·042)	499·60 (0·027)	498·80 (0·030)	498·60 (0·040)
Fruit juice**						
Orange	Ascorbic acid	–	102·00 (0·020)	103·40 (0·048)	–	103·80 (0·028)
Lemon	Ascorbic acid	–	178·50 (0·062)	177·90 (0·052)	–	179·00 (0·030)
Tomato	Ascorbic acid	–	25·40 (0·040)	25·00 (0·022)	–	26·20 (0·022)
Aminodrip	Methionine	316·80	309·80 (0·060)	310·00 (0·020)	312·20 (0·058)	
INH tablets						
Isokin (Parke-Davis)	Isonicotinic acid hydrazide	300	294·00 (0·022)	296·20 (0·018)	–	298·90 (0·040)
Isonex (Pfizer)	Isonicotinic acid hydrazide	100	98·30 (0·020)	99·00 (0·042)	–	101·20 (0·032)

*Average of five determinations; **two fruits in each case were analysed; standard deviations are given in parentheses

4.5 *Determination of methionine present in aminodrip*

In order to assess the possible analytical application of the proposed method, the effect of some of the amino acids that accompany methionine in aminodrip has been studied and at their indicated levels below do not interfere in 1 M sulphuric, hydrochloric or 2 M phosphoric acid. Methionine present in aminodrip has been determined and the results given in table 4 compare with the manufacturer's specification [composition/100ml of aminodrip: L-arginine (495·00 mg), L-histidine (33·75 mg), L-cystine (1229·25 mg), L-tyrosine (56·25 mg), L-tryptophan (0·55 mg), L-cysteine (5·60 mg), L-methionine (316·80 mg), glycine (1650·00 mg), L-threonine (129·30 mg), L-leucine (756·75 mg), L-isoleusine (106·80 mg), L-valine (157·50 mg), L-alanine(675·00 mg), L-proline (345·00 mg), L-serine (165·00 mg), L-hydroxyproline (315·00 mg), L-aspartic acid (360·00 mg), L-glutamic acid (450·00 mg) and L-phenylalanine (253·05 mg)].

4.6 *Titration of isonicotinic acid hydrazide*

Isoniazid (INH) is the most important drug in the treatment of tuberculosis which induced many investigators to work out methods for its rapid and accurate determination. Among them, the titrimetric methods are frequently used which are based on the oxidation of INH quantitatively to nicotinic acid and nitrogen involving a four electron change. Some of the indicators used so far for the determination of INH using CAT are unsatisfactory for one or other reason. For example, methyl red and methyl orange work in phosphoric acid medium only.

In the titrations of 0·05–0·01 N INH, BAPP gives sharp and irreversible colour change from colourless to blue via pink in 0·5–2·0 M sulphuric, 0·5–1·8 M hydrochloric or 2·5–3·5 M phosphoric acid solution containing potassium bromide. The colour is stable for 3 min in all the acid media. At lower and higher acidities premature and overstepping end-points respectively were obtained.

The following amounts of tablet diluents and excipients do not interfere in the determination of 50 mg of INH: citric acid (700 mg), gelatin (75 mg), starch (350 mg), oxalic acid (650 mg), glucose (600 mg), talc (200 mg), stearic acid (150 mg), alcohol (5 ml). The INH content in tablets like Isokin (Parke-Davis) and Isonex (Pfizer) was determined. The results presented in table 4 were compared with those found by the published method[17]. Also, the found values agreed well with the claimed values on the label in all the tablets.

4.7 *Titration of phenylhydrazine hydrochloride*

Bromine oxidizes phenylhydrazine hydrochloride to benzene diazonium chloride involving four electron changes. BAPP gives sharp and reversible end-points in the titration of 0·05–0·01 N phenylhydrazine hydrochloride. There is a brilliant colour change from colourless to blue in 0·2–1·0 M sulphuric acid, 0·2–1·5 M hydrochloric acid or 1·0–3·0 M phosphoric acid solution containing potassium bromide. The end-point colour is stable for 3 min. BAPP gives late and premature end-points respectively at lower and higher acidities. Premature end points were obtained in acetic acid medium. BAPP has advantages over indigocarmine in that it (i) gives sharper end-points and more accurate values, (ii) functions in three acid media while indigocarmine functions in hydrochloric acid medium only, and (iii) BAPP is used as an indicator at laboratory temperature, while indigocarmine works only at elevated temperature.

4.8 *Determination of biotin*

Biotin (vitamin H) is necessary for the growth of animals. The important role of biotin as the prosthetic group of certain carboxylating, transcarboxylating or decarboxylating enzymes is well known. Some physico-chemical biotin assays have been reported, but they have not become widely accepted for routine biotin assays. A survey of literature has revealed that only a couple of titrimetric procedures are available for the determination of biotin[19,20]. The titrimetric procedure using iodine trichloride as an oxidant involves a tedious extraction step using an organic solvent and hence the authors believed that it is worthwhile to develop more simple, rapid, sensitive, accurate and inexpensive methods for the determination of biotin. Therefore, BAPP has been proposed as a redox indicator

for the oxidimetric estimation of biotin using CAT. Bromine oxidizes sulphide group of biotin to sulphoxide [20].

$$C_{10}H_{16}\text{–}S\text{–}N_2O_3 + Br_2 + H_2O \rightarrow C_{10}H_{16}\text{–}\overset{\overset{O}{\|}}{S}\text{–}N_2O_3 + 2HBr.$$

In the titration of 0·01–0·0025 N biotin, BAPP gives sharp and irreversible end-points accompanied by a brilliant colour change from colourless to blue via pink in 0·2–2·0 M sulphuric acid or hydrochloric acid or 1·0–3·5 M phosphoric acid solution containing potassium bromide. The end-point colour is stable for 25 min in all the acid media. Late and premature end-points were obtained respectively at lower and higher acidities. Acetic acid was not suitable for this titration. The proposed method is convenient, rapid and precise. No reports regarding the use of internal redox indicators in the titration of biotin against CAT are available to date. Hence the authors claim that BAPP could become the first of its kind to use as redox indicator in the titration of biotin with CAT.

4.8a *Potentiometric titration of biotin:* In the potentiometric titration of 0·01–0·0025 N biotin with CAT, the presence of 1–3% potassium bromide is desirable as it stabilises the potentials and also increases the potential break. The time required for the attainment of equilibrium potential is about 2 min and the potential break is 307 mV with 0·1 ml of 0·01 N CAT solution. Titration in 0·5–1·0 M sulphuric acid or 0·5–1·5 M hydrochloric acid or 2·5–3·0 M phosphoric acid medium gives accurate and reproducible values. An increase in the acid concentration gave higher titres. In the absence of potassium bromide, potentials of the systems were not stabilized. The results presented in table 3 compare favourably with those of the potentiometric determination of biotin with CAT.

The effect of bromide concentration on the determination of ascorbic acid, methionine, isonicotinic acid hydrazide, phenylhydrazine hydrochloride and biotin was examined and the minimum amount of KBr required in the titrations of 0·05–0·01 N reductant is 0·3–0·5 g in a total volume of 60 ml or in the titrations of 0·01–0·005 N reductant is 0·20–0·40 g in a total volume of 35 ml. Higher concentrations (up to 3%) do not affect it and lower concentrations result in sluggish end-points. At least 0·2 ml of 0·1% BAPP in a total volume of 60 ml or 0·05 ml of 0·1% BAPP in a total volume of 35 ml was necessary for proper indicator action. Higher concentrations of the indicator > 0·4 ml or > 0·1 ml give higher titre values and lower concentrations give sluggish end-points. The average indicator correction was found to be 0·05 ml of 0·05 N CAT for 0·2 ml of 0·1% BAPP or 0·2 ml of 0·005 N CAT for 0·1 ml of 0·1% BAPP indicator. BAPP has advantages over phenothiazine indicators in that it gives sharper end-points and more accurate titre values and has less indicator correction. Further, the amount of BAPP required for the indicator action is very little (0·2 ml of 0·1%) compared to phenothiazines (2 ml of 0·1%).

During titration of ascorbic acid, methionine, INH, phenylhydrazine hydrochloride or biotin, it was found that BAPP undergoes one-electron oxidation to give a radical cation which is pink in colour. The radical cation undergoes further one-more electron oxidation to give a blue coloured dication at the equivalence point. The redox and the transition potentials for BAPP have been determined and the values lie within the potential break in the potentiometric titrations of reductants with CAT in the presence of sulphuric acid, hydrochloric acid or phosphoric acid. Further, the formal and the transition potentials

indicate that BAPP serves as a good indicator in the titration of reductants with CAT. In the titration of methionine, INH and biotin, if the indicator is added at the beginning of the titration, no clear end-point was obtained because of the partial destruction of the indicator. When titrating unknown samples, an approximate titre was found by adding 1–2 ml of 0·1% BAPP at the beginning of the titration and then the correct titre was found by adding the indicator near the end-point.

The titration results given in table 3 for different reductants examined are typical and are considered to compare well with the results obtained by other available titrimetric methods for ascorbic acid [17], methionine [21], INH [17], phenylhydrazine hydrochloride [17] and biotin (potentiometric method).

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