convulsions and to prolong the latency to the first myoclonic jerk induced by Ro 5-3663 (10 mg kg^{-1}). Vellucci & Webster (1983) also found Ro 15-1788 (25, 50 mg kg^{-1}) effective against convulsions caused by Ro 5-3663 at 10 mg kg^{-1}, but not those caused by higher doses. Both of these findings contrast with the failure of Green et al (1982) to find any change in the seizure threshold to an intravenous infusion in rats of Ro 5-3663, following a 15 min pretreatment with Ro 15-1788 (10 mg kg^{-1} i.p.). This difference may be the result of different methodology or species used, but it is striking that the seizure threshold in rats, 10·7 mg kg^{-1} Ro 5-3663 is close to the one we found in our mice. The ability to see anticonvulsant effects against Ro 5-3663 more easily than picrotoxin is further evidence of the latter being a more powerful convulsant.

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Application of orthogonal functions to spectrophotometric analysis of the preservatives benzylalcohol, phenol and parabens in aqueous cyanocobalamin solutions

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The orthogonal polynomials P_3 and P_2, 12 and 8 points, at 2 nm intervals over certain wavelength ranges were used for the spectrophotometric analysis of benzylalcohol, phenol or parabens in aqueous cyanocobalamin solutions. The 12 point-methods proved to be more sensitive and direct and to have sufficient accuracy and precision.

Benzylalcohol, phenol and parabens are preservatives compatible with cyanocobalamin in aqueous solutions. They are currently determined by gas chromatography (USP XX; Hrivnak & Macak 1971; Johnson & Venturello 1971; Douglas 1972). Previous methods include spectrophotometric (Glenn 1960; Elvidge & Peutrell 1961) and colorimetric (Gibbs 1927; Emerson 1943; Johnson & Savidge 1958) methods, which have limitations in the presence of interfering substances. Glenn (1963) applied orthogonal functions to the spectrophotometric assay of phenol and adrenaline in a parenteral solution.

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We have applied orthogonal functions to the determinations of benzylalcohol, phenol and parabens in aqueous cyanocobalamin solutions since it has the advantage of being a direct fairly sensitive method that does not require expensive apparatus. Optimum conditions (Glenn 1963 Abdine et al 1971) were determined and computed from the absorption spectra of benzylalcohol, phenol, parabens and cyanocobalamin in 0·05 M sulphuric acid, scanned individually at 1 nm intervals (Fig. 1).

Materials and methods

Materials used were cyanocobalamin BP, phenol and benzylalcohol, both BDH laboratory reagents, the latter being further purified by shaking with sodium metabisulphite and filtering; methylparaben and propylparaben were of BP quality.

Solutions prepared: Aqueous cyanocobalamin solutions (0·01–0·1% w/v) were freshly prepared and contained

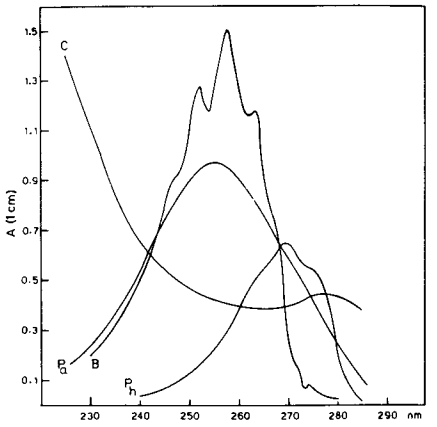

FIG. 1. U.v. spectrum of benzylalcohol (B), phenol (Ph), parabens (Pa) and cyanocobalamin (C), in 0·05 M sulphuric acid.

one of the following bacteriostats: benzylalcohol (0·7–3% w/v) phenol (0·13–0·78% w/v) or parabens mixture (consisting of methylparaben 5 parts, with propylparaben 3 parts) (0·05–0·1% w/v).

Assay. Dilute a 2·0 ml aliquot of each prepared solution and bring up to volume with 0·05 M sulphuric acid in a 100 ml volumetric flask. Further dilute with water, if necessary, to obtain readings within the chosen wave length range for each prepared diluted solution as follows:

(a) For solutions of cyanocobalamin containing benzylalcohol: measure the absorbance of the suitably diluted solution containing about 40 mg% benzylalcohol over the wavelength range 240–262 nm, 12 points (λm = 251 nm). Calculate the P_3 coefficient using the following equation (Fisher & Yates, 1953):

P_3 coefficient (12 point) unnormalized = $(-33)\,A_0 + (+3)\,A_1 + (+21)\,A_2 + (+25)\,A_3 + (+19)\,A_4 + (+7)\,A_5 + (-7)\,A_6 + (-19)\,A_7 + (-25)\,A_8 + (-21)\,A_9 + (-3)\,A_{10} + (+33)A_{11}$

Where A_0; A_1, etc. represent absorbance at each wavelength interval.

Find the concentration of benzylalcohol in the solution from the P_3 coefficient of a separately prepared benzylalcohol, 40 mg% in 0·05 M sulphuric acid solution (Glenn 1963).

(b) For solutions of cyanocobalamin containing phenol: measure the absorbance of the diluted test solution containing about 2–4 mg% phenol over the wavelength range of 260–282 nm (λ m 271 nm), 12 points, or over the wavelength range of 266–280 nm (λ m 273), 8 points. Calculate the P_2 coefficient and find the concentration of phenol using the following equations:

P_2 coefficient unnormalized (12 points) = $(+55)\,A_0 + (+25)\,A_1 + (+1)\,A_2 + (-17)\,A_3 + (-29)\,A_4 + (-35)\,A_5 + (-35)\,A_6 + (-29)\,A_7 + (-17)\,A_8 + (+1)\,A_9 + (+25)\,A_{10} + (+55)\,A_{11}$

P_2 coefficient unnormalized (8 points) = $(+7)A_0 + (+1)\,A_1 + (-3)\,A_2 + (-5)\,A_3 + (-5)\,A_4 + (-3)\,A_5 + (+1)\,A_6 + (+7)\,A_7$

(c) For solutions of cyanocobalamin containing parabens: measure the absorbance of the diluted solution containing from 0·6–0·8 mg% parabens over the wavelength range of 246–268 nm (λ m 257 nm), 12 points, or over the wavelength range of 246–260 (λ m 253 nm) 8 points. Calculate the P_2 and coefficient and find the concentration of parabens as mentioned before.

Also, for all prepared solutions of cyanocobalamin, at an appropriate dilution step, measure absorbance at 361 nm, in order to determine concentration of cyanocobalamin with reference to a standard solution of cyanocobalamin.

Results and discussion

When the orthogonal polynomial P_3, 12 points at λ m = 251 nm were used, the assay results for benzylalcohol in the presence of cyanocobalamin as irrelevant absorbance gave a recovery of 100·65% ± 1·3 (P = 0·05, n = 6), providing the ratio of benzylalcohol to cyanocobalamin was not less than 19 : 1. Below this ratio the assay of benzylalcohol is dependent on the cyanocobalamin concentration.

Comparison of the orthogonal function method with a spectrophotometric method for benzylalcohol at two wavelengths, 256 and 361 nm, revealed in the latter case a recovery of 100·43% ± 2·77 (P = 0·05, n = 4) denoting a lower precision. The results were obtained after measurement of the absorbance of a reference cyanocobalamin solution (4 mg%) at the two wavelengths and of a reference benzylalcohol solution (40 mg%) in 0·05 M sulphuric acid at 256 nm, which did not show an absorbance at 361 nm.

With the orthogonal polynomial P_2, phenol, in the presence of cyanocobalamin as an irrelevant impurity, was determined and recovered to 100·13% ± 2·0 (P = 0·05, n = 7) (12 points) at λ m 271 nm and 100·722% ± 1·42 (P = 0·05, n = 5) (8 points) at λ m 273 nm. The results were obtained from varying ratios of phenol to cyanocobalamin in the range of 4–7·5 : 1. In comparison, a spectrophotometric determination of phenol in cyanocobalamin solution at two wavelengths 270 and 361 nm resulted in a recovery of 99·66% ± 1·4 (P = 0·05; n = 6), indicating a more accurate result with the twelve point method.

Application of the orthogonal polynomial P_2 to the assay results of parabens in the presence of cyanocobalamin as irrelevant absorbance gave a recovery of 97·78% ± 3·3 (P = 0·05; n = 5), 12 points at λ m 257 nm and 98·3% ± 8·9 (P = 0·05; n = 5), 8 points at λ m 253 nm in a ratio ranging from 0·6–2·4 : 1 of parabens to cyanocobalamin. Thus more precise results were obtained with the twelve point-method.

However, the recovery of parabens in a ratio range from 0·9–2 : 1 of cyanocobalamin was 98·95% ± 2·9 (P = 0·05; n = 5) 12 points, indicating more accurate

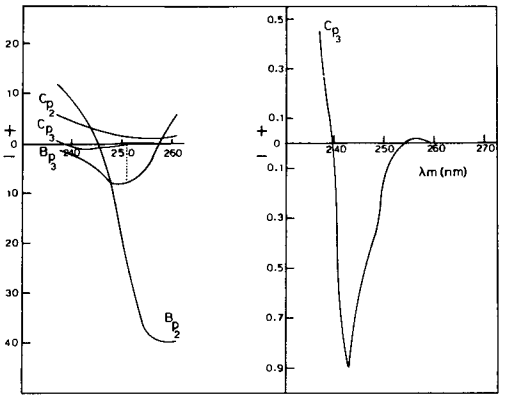

FIG. 2. P_2 and P_3 convoluted curves (unnormalized), 12 points of 40 mg% and 1 mg% of benzylalcohol (B) and cyanocobalamin (C) respectively.

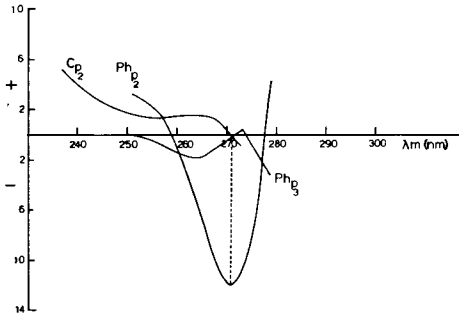

FIG. 3. P_2 and p_3 convoluted curves (unnormalized), 12 points of 1 mg% of phenol (Ph) and cyanocobalamin (C).

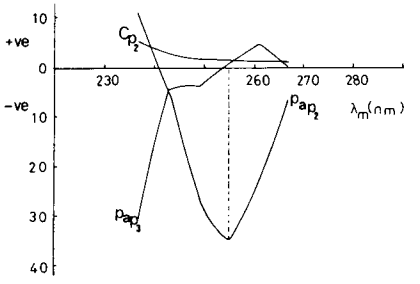

FIG. 4. P_2 and P_3 convoluted curves (unnormalized, 12 points) of 1 mg% of parabens (pa) and cyanocobalamin HCl (C).

results attributable to a lower contribution from cyanocobalamin. By application of the colorimetric method described by Johnson & Savidge (1958), to parabens in presence of cyanocobalamin, a large sample size and a prehydrolysing step was required, which led to a recovery of 99·37% ± 2·9 ($P = 0{\cdot}05$; n = 4). Figs 2, 3 and 4 represent the unnormalized P_2 and P_3 convoluted curves, 12 points of the preservatives and cyanocobalamin. The optimum wavelength range and intervals were chosen to maximize P_j of each preservative and minimize P_j of cyanocobalamin. As shown in Table 1, and in accordance to Glenn's theory (Wahbi 1967), the comparative coefficients q_j of the preservatives, with the exception of benzylalcohol, exceed 140×10^{-3}.

Table 1. Comparative coefficients calculated at the optimum set of wavelengths, at 2 nm intervals.

Solution	Strength mg%	Pts	P*	$P_j \times 10^3$	$q_j \times 10^3$
Benzylalcohol	40	12	P_3	−1·445	103·7
Phenol	2	12	P_2	−1·982	217·2
	4	8	P_2	−14·48	187·7
Parabens	1	12	P_2	−2·674	293·0
	2	8	P_2	−19·10	247·6

* Polynomial.

A spectrophotometric determination of cyanocobalamin at 361 nm in all solutions containing the preservatives resulted in a recovery of 100·15% ± 0·1 ($P = 0{\cdot}05$; n = 9).

To conclude, the preservatives, benzylalcohol, phenol or parabens in aqueous cyanocobalamin solutions could be spectrophotometrically determined by application of orthogonal functions using the appropriate polynomial and set of wavelengths. The method was fairly rapid, sensitive and did not require a large sample or expensive apparatus.

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