Table V—Authentic Recoveries, Acetonitrile Sample Preparation Solvent

Formulation Type[a]	Recovery of Label Claim, %	
Nitroglycerin		
1	97.5	100.8
2	101.1	100.5
3	100.3	100.4
4	98.3	101.8
5	101.3	99.7
	average	100.1
	CV[b] 1.25%	
Isosorbide Dinitrate		
3	100.6	101.6
5	97.8	98.3
6	102.0	100.6
7	100.0	99.2
	average	100.0
	CV 1.50%	
Pentaerythritol Tetranitrate		
6	99.0	99.2
7	99.9	100.1
8	98.2	99.8
	average	99.4
	CV 0.72%	

[a] Formulation types correspond by number to manufacturer numbers in previous tables. [b] Coefficient of variation.

magnesium stearate, talc, dried malt syrup, microcrystalling cellulose, sodium starch glycolate, calcium sulfate, stearic acid, starch, colloidal silica, lactose, polyethylene glycol, guar gum, FD&C blue No. 1 and FD&C yellow No. 5. Placebo mixtures without active ingredient or internal standard gave no peaks past the solvent front. Excipient peaks would not interfere with the detection of incompletely nitrated impurities.

The results of assays of authentic mixtures containing active ingredient are given in Table V. The average recoveries of 99.4–100.1% with 0.72–1.50% *CV* indicate that the accuracy and reproducibility of the method are acceptable for the analysis of this type of product. The results of the spiked authentic samples demonstrate that 60-min shaking time is adequate to extract the drug. Since this is compatible with requirements of chromatograph start-up and equilibration, shorter times were not investigated.

Linearity was tested using authentic mixtures. For nitroglycerin linearity was demonstrated from 0.2 to 0.8 mg/ml, representing 40–160% of label declaration. A correlation coefficient of 0.9990 was obtained (10) from the analysis of 0.2-, 0.4-, 0.5-, 0.6-, and 0.8-mg/ml samples. For isosorbide dinitrate linearity was demonstrated from 0.27 to 0.8 mg/ml, representing 54–160% of label declaration. A correlation coefficient of 0.9998 was obtained (10) by measuring 0.27, 0.45, 0.55, 0.63, and 0.80 mg/ml. For pentaerythritol tetranitrate linearity was demonstrated from 0.5 to 1.5 mg/ml, representing 50–150% of label declaration. A correlation coefficient of 0.9999 was obtained (10) by measuring 0.5, 0.78, 1.0, 1.24, and 1.5 mg/ml.

It appears that reverse-phase HPLC, after sample preparation with acetonitrile, is a useful method for the analysis of nitroglycerin, isosorbide dinitrate, and pentaerythritol tetranitrate sustained-release tablets and capsules. Since nitroglycerin hydrolysis products were recently demonstrated to elute substantially prior to nitroglycerin in this system (11), the method is anticipated to be stability indicating for nitroglycerin.

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Titrimetric Determination of Ascorbic Acid with 2,6-Dichlorophenol Indophenol in Commercial Liquid Diets

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Abstract ◻ The titrimetric determination of ascorbic acid in the presence of a variety of potentially physically and chemically interfering species in commercial liquid diets is presented. The titrant and indicator was a solution of 2,6-dichlorophenol indophenol. Iron(II), copper(II), cysteine, glutathione, sulfite, and tin(II) do not interfere.

Keyphrases ◻ Ascorbic acid—titrimetric determination with 2,6-dichlorophenol indophenol in commercial liquid diets ◻ 2,6-Dichlorophenol indophenol—titrimetric determination of ascorbic acid in commercial liquid diets ◻ Titrimetric determination—ascorbic acid determination with 2,6-dichlorophenol indophenol in commercial liquid diets

Ascorbic acid frequently appears in multicomponent media, except in simple synthetic preparations such as ascorbic acid injection (1). In commercial liquid diets, for example, it is accompanied by proteins, amino acids, saccharides, lipids, and minerals (2). Methods involving oxidation of ascorbic acid are complicated by oxidizable metal ions, notably iron(II) and tin(II) (3).

The titrimetric oxidation of the two enolic groups of

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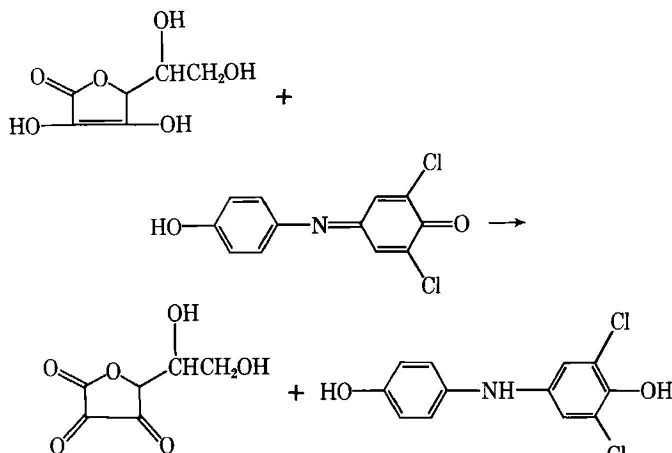

Scheme I—Oxidation of ascorbic acid by 2,6-dichlorophenol indophenol

ascorbic acid to the two keto groups of dehydroascorbic acid by 2,6-dichlorophenol indophenol (Scheme I) lacks specificity in all but the most chemically inert systems. Other traditional methods are available. Microbiological assays are specific, but they are lengthy and suffer from poor precision. A popular colorimetric procedure (4) and a modification using *p*-nitroaniline are sensitive to impurities and unresponsive at low ascorbic acid concentrations. An existing microfluorometric assay (5) does not distinguish between ascorbic acid and dehydroascorbic acid; this may be a serious shortcoming in stability studies, since dehydroascorbic acid is the principal degradation product of ascorbic acid. Methods for selective ascorbic acid determination appear to require extensive sample clean-up (6) or a sophisticated chromatographic system with selective detection such as high-performance liquid chromatography (HPLC) (7, 8), ion-exchange chromatography (9), electrochemical detection (10), amperometric detection (11), or differential-pulse polarography (12).

Although the HPLC methods mentioned are sensitive, frequently they are unable to resolve ascorbic acid from the water-soluble B vitamins. The more sophisticated detectors may not be available in many laboratories. The speed and accuracy of a titrimetric method is especially attractive where ascorbic acid analysis is done on a large number of samples or on a routine basis. The purpose of this report is to present a modification of the standard 2,6-dichlorophenol indophenol titration such that it may be run in the presence of a large number of potential physical and chemical interferences present in commercial liquid diets.

BACKGROUND

Almost 50 years ago, the desirability of increasing the specificity of the 2,6-dichlorophenol indophenol titration to exclude the ferrous ion and glutathione was well understood (13–15). The titrimetric method was then sufficiently well developed to spawn its use in an impressive list of media including fruit and vegetable juice, milk, body tissues, cerebral fluid, plant extracts, and animal organs (16, 17). The animal and vegetable media offer few interferences, but biological systems often contain enough oxidizable metal ions, notably ferrous ions, to interfere. Since the 1940s, the 2,6-dichlorophenol indophenol titration has been unearthed and the ferrous ion interference eliminated by carrying out the titration in acid. Hence, the ferrous ion interference was eliminated by titrating in acetic acid (18). The interference also was removed by titrating in hydrochloric acid solution (19), and hydrochloric acid–acetate mixtures (20). Aqueous acetic acid solutions also were used to eliminate ferrous ion interference

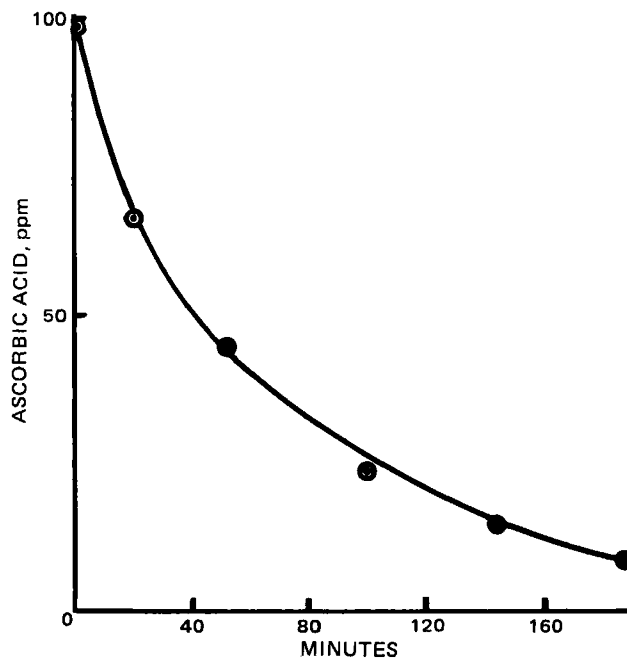

Figure 1—*Degradation of ascorbic acid in an unmasked sample (pH 5.0).*

(21, 22). In a two-step titration of ferrous ion and ascorbic acid, the ferrous ion was masked by an aqueous succinic or adipic acid solution (23). Despite these successful attempts to mask the ferrous ion during the ascorbic acid determination, extension of the method to include other redox interferences is absent from the chemical literature.

An example of a redox interference is the presence of a reducible species in the sample such as cupric or ferric ions. The metal ions are reduced, and the ascorbic acid is oxidized yielding erroneously low ascorbic acid values. The reaction, $e^- + Cu^{2+} \rightarrow Cu^+$, occurs stoichiometrically with the ascorbic acid oxidation, and the cupric ion has been used to determine ascorbic acid (24). The ferric ion interference may be masked in some samples with the tartrate ion.

Oxidizable metal ions and organic species, notably the mercaptans, cysteine and glutathione, interfere and yield high ascorbic acid assay values. The 2,6-dichlorophenol indophenol reacts with both the ascorbic acid and the oxidizable mercaptan. These interferences may be avoided by low pH solutions, and weak organic acids may be preferred since ascorbic acid is destroyed in very acidic solutions.

Sulfur dioxide interference has been eliminated by addition of acetone, hydrogen peroxide, or formaldehyde. Strongly colored solutions have been decolorized with tablets[1] (25) to allow a visual endpoint.

The standard 2,6-dichlorophenol indophenol titration is seen to be usable when other species in solution are not easily oxidized or reduced. Prevention of one oxidation reaction, $Fe^{2+} \rightarrow Fe^{3+} + e^-$, may be accomplished by dissolving the sample in a solution of a weak acid. The standard titration is not selective enough for analysis of biological fluids (since they often contain oxidizable species such as cysteine and glutathione), soft drinks that contain the sulfite ion, and vitamin formulations containing the stannous ion. None of the previous reports (18–23) attempted to modify the 2,6-dichlorophenol indophenol titration to select ascorbic acid in the presence of any oxidizable species except ferrous ion. The present report investigates sample handling conditions which would mask cysteine, glutathione, sulfite, and tin(II) and, hence, extend the utility of 2,6-dichlorophenol indophenol titration to systems where selectivity is important.

EXPERIMENTAL

Reagents—All chemicals were analytical reagent grade unless otherwise noted.

Procedure—A sample containing ~10 mg of ascorbic acid was weighed and transferred to a 125-ml Erlenmeyer flask having a ground-glass stopper. In addition to ascorbic acid, the samples contained glucose oligosaccharides, L-glutamine, L-aspartic acid, glycine, L-phenylalanine,

[1] Boehringer.

Table I—Titrimetric Assay of Various Commercial Liquid Diets

Sample	Ascorbic Acid[a], ppm	Colorimetric Assay[b]
1	347	325
2	285	277
3	251	240
4	237	240
5	160	
6	166	

[a] Label value for all products was 160 ppm of ascorbic acid. [b] Ref. 30.

L-proline, L-leucine, L-lysine hydrochloride, L-alanine, L-arginine hydrochloride, magnesium gluconate dihydrate, sodium citrate dihydrate, L-methionine, L-valine, L-threonine, L-isoleucine, L-serine, calcium glycerophosphate, L-histidine hydrochloride monohydrate, sodium glycerophosphate, potassium citrate monohydrate, safflower oil, L-tryptophan, potassium chloride, potassium sorbate, L-tyrosine, polyoxyethylene sorbitan monooleate, ferrous gluconate dihydrate, choline bitartrate, zinc acetate dihydrate, α-tocopherol acetate, niacinamide, manganese glycerophosphate, D-calcium pantothenate, cupric citrate, ascorbyl palmitate, pyridoxine hydrochloride, riboflavin phosphate sodium salt, vitamin A acetate, thiamine hydrochloride, folic acid, *d*-biotin, potassium iodide, phytonadione, ergocalciferol, and cyanocobalamin. About 20–40 ml of 1.5 *N* acetic acid was added. The sample was titrated immediately with the titrant consisting of 0.2 g of 2,6-dichlorophenol indophenol and 0.2 g of sodium bicarbonate and distilled water sufficient to make 1000 ml of solution. The endpoint was a distinct pink color which lasted for 30 sec and was more intensely colored than a phenolphthalein endpoint. Similarly, a semimicro titration was performed. For a 5-ml buret, the titrant was prepared from 1.2 g of 2,6-dichlorophenol indophenol and 1.2 g of sodium bicarbonate to make 1 liter of solution. The titrant was standardized by titration against a standard solution of 100 mg of USP ascorbic acid[2] in 100 ml of 1.5 *N* aqueous acetic acid. Two milliliters of this standard solution was pipeted and titrated for the titrant standardization in both the macro and semimicro titrations.

Wet additions of cysteine, glutathione, sodium sulfite, and tin(II) chloride were made to a final concentration in the sample solutions of 66.6–333 μg/ml. The samples were then assayed for ascorbic acid using the semimicro titration procedure.

RESULTS AND DISCUSSION

Some confusion exists in the literature over the nomenclature for the determination of ascorbic acid by oxidation to dehydroascorbic acid. Some investigators choose ascorbic acid, L-ascorbic acid, vitamin C, or total vitamin C as the species determined. Since the 2,6-dichlorophenol indophenol oxidation does not distinguish between D and L forms, and since vitamin C is defined (26) as L-ascorbic acid, the generic term ascorbic acid may be less misleading. Total vitamin C is defined as the arithmetic sum of the L-ascorbic acid plus dehydroascorbic acid content of the sample. Dehydroascorbic acid has the same molar biological (antiscorbutic) activity as ascorbic acid (26), and, hence, assays of biological origin usually attempt to determine total vitamin C. Any assay specific for ascorbic acid does not necessarily measure the biological activity, since some or all of the ascorbic acid in the sample may have been oxidized to dehydroascorbic acid. Conforming to official USP nomenclature, the species determined by the described procedure is ascorbic acid (D and L forms). Dehydroascorbic acid (and, therefore, the total vitamin C or biological activity) is neither determined nor does its presence interfere. If an assay for total vitamin C is desired, prior reduction of dehydroascorbic acid with hydrogen sulfide may be possible (27, 28).

In many solid samples, the formation of dehydroascorbic acid follows dissolution in water. In standard solutions, the aerial ascorbic acid to dehydroascorbic acid conversion is slow, requiring 24 hr for 0.3% dehydroascorbic acid to be formed (29). Inert atmospheres of carbon dioxide, nitrogen, and hydrogen have been employed to slow degradation further. Although it would appear that no special sample handling is required, even minute amounts of ferric and cupric ions present in samples, strongly catalyze the ascorbic acid oxidation (2). In Fig. 1, considerable oxidation of ascorbic acid is seen to occur in the samples used in this study, even in an acidic medium. Even when the ferric ion has been eliminated by complexation or masking as a direct interference, the ion is still capable of catalysis of the ascorbic acid to dehydroascorbic acid conversion. When the dissolution medium is 1.5 *N* acetic acid, no degradation is observed for at least the first 5 min.

[2] Aldrich Chemical Co., Milwaukee, Wis.

Table II—Dry Recoveries of Ascorbic Acid from Placebo

Milligrams Added	Milligrams Found	Recovery,[a] %
0.500	0.500	100
0.500	0.520	104
1.00	1.02	102
1.00	1.00	100
1.50	1.47	98
1.50	1.46	97
1.56	1.53	98
1.56	1.53	98
1.56	1.53	98
1.56	1.52	97
1.95	1.92	98
1.95	1.91	98
1.95	1.92	98
1.95	1.90	97
2.34	2.36	101
2.34	2.37	101
2.34	2.35	100
2.34	2.35	100

[a] Average = 99.2%. *SD* = 1.95. *RSD* = 1.96%.

Initial attempts to increase the specificity of the 2,6-dichlorophenol indophenol oxidation of ascorbic acid using 0.1 *M* hydrochloric, nitric, and sulfuric acid media for dissolution were unsuccessful. The samples containing the ferrous ion yielded an assay equal to ascorbic acid plus the ferrous ion content. Five percent aqueous trichloroacetic acid was then used to dissolve some of the samples. The assay values were time-dependent, *i.e.*, the longer the titration time, the lower the ascorbic acid content. *m*-Phosphoric acid (0.1 *M*), sulfuric acid, and oxalic acid were tested, since they have been reported to increase the specificity of the reaction (28). None yielded satisfactory recoveries when sulfite was present. After trials with several mixed solvent and weak organic acid combinations, aqueous acetic acid was found to be the most applicable sample medium. The most dilute solution, which consistently masked ferrous ions, stannous ions, sulfite, cysteine, and glutathione was 1.5 *N* CH_3CO_2H. This dissolution medium was also found to mask cupric ions and other excipients listed in *Experimental*. Several commercial liquid diets were assayed titrimetrically and the results appear in Table I. Since commercial liquid diets frequently contain a large excess of ascorbic acid, some formulations were also assayed by the colorimetric determination of the 2,4-dinitrophenylhydrazine derivative of dehydroascorbic acid (30). The two methods are in good agreement.

The selectivity of the titrimetric procedure presented here compares favorably with other oxidative titrations. Titration with *N*-bromosuccinimide, for example, is complicated by cysteine, glutathione, and sulfite (31). The selectivity of the basic chloramine T procedure (32) is limited to samples without sulfite and sulfhydryl compounds, thiosulfates, sulfides, or tin(II). The modified 2,6-dichlorophenol indophenol determination is seen to be highly selective when compared with other titrimetric procedures.

The utility of the 2,6-dichlorophenol indophenol titration of ascorbic acid has been extended to systems of a biological composition in the presence of iron(II), copper(II), cysteine, glutathione, sulfite, tin(II), and other redox interferences. The data for 18 dry recovery samples are presented in Table II. The ascorbic acid was introduced to the placebo at six levels ranging from 0.5–2.34 mg. The average recovery was 99.2% with a relative standard deviation of 1.96%. For the precision of the procedure, 11 samples ranging from 4.34–6.48 g were assayed. All samples

Table III—Precision of Ascorbic Acid Titrimetric Assay

Sample, g	μg of Ascorbic Acid/g,[a]
4.34	101.0
4.17	97.5
4.34	101.0
5.37	99.9
5.25	98.1
5.47	102.0
5.48	102.0
6.56	102.0
6.53	102.0
6.51	101.0
6.48	101.0

[a] Label value was 97.5 μg ascorbic acid/g sample; mean = 101 μg/g. *SD* = 1.57 μg/g. *RSD* = 1.56 μg/g.

Table IV—Recoveries of Ascorbic Acid in the Presence of Known Interferences

	μg/ml	Ascorbic Acid Added, μg[a]	Found, μg[a]	Recovery, %
Cysteine	66.6	666	663	99.6
	133.0	666	672	101.0
	200.0	666	667	100.0
	266.0	666	673	101.0
	333.0	666	671	101.0
Glutathione	66.6	666	669	100.0
	133.0	666	665	100.0
	200.0	666	660	99.2
	266.0	666	661	99.3
	333.0	666	672	101.0
Sulfite	66.6	666	666	100.0
	133.0	666	668	100.0
	200.0	666	671	99.2
	266.0	666	660	99.2
	333.0	666	663	99.6
Tin(II)	66.6	666	656	98.5
	133.0	666	667	100.0
	200.0	666	660	99.0
	266.0	666	662	99.4
	333.0	666	667	100.0

[a] Mean = 665 μg. *SD* = 5.0 μg. *RSD* = 0.75%.

were obtained from the same lot and the theoretical ascorbic acid content was 97.5 μg/g. The samples displayed good homogeneity and assayed at an average of 101 μg of ascorbic acid/g of sample or 104% of label value. The data appear in Table III. In Table IV data are presented for the recovery of ascorbic acid in the presence of known redox interferences. In each case a wet spike of the interference was made to a standard containing 666 μg of ascorbic acid. The resulting solution was then 333 μg/ml in ascorbic acid and from 66.6–333 μg/ml (20–100% of the ascorbic acid concentration) interference. The titration was also performed without the acetic acid masking solution, and in each case, the recoveries were markedly <100%. Dissolution of samples in 1.5 *M* acetic acid masks commonly encountered interferences and allows the use of a titrimetric procedure where more sophisticated methods were previously required.

The simplicity of the method cannot be overstated. The titrant is the only reagent required for the titration (since the titrant is also the indicator) and is stable for at least 1 month. The sample preparation consists of shaking with 1.5 *M* acetic acid for several seconds. Other titrimetric procedures not only are more time consuming for the determination itself, but require special procedures or unstable reagent. Reduction of the 1,2-enediol group by *N*-bromosuccinimide, for example, requires titration with an unstable reagent. Titration of ascorbic acid with chloramine T in some cases requires a carefully prepared sodium tetrathionate solution which is thermally and photochemically degradable. Although a recent report (32) describes the determination of ascorbic acid with chloramine T in the presence of sulfhydryl compounds or sulfite, it does not outline a procedure for safely distilling the acrylonitrile. Some analysts may be unwilling to distill as highly toxic, flammable, and explosive (33) a liquid as acrylonitrile. The oxidation of the 1,2-enediol group by 2,6-dichlorophenol indophenol appears to be the fastest and safest titrimetric determination of ascorbic acid presently available.

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