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Determination of thymol in halothane anaesthetic preparations by high-performance liquid chromatography

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Abstract: A high-performance liquid chromatographic (HPLC) procedure has been developed for the isolation and quantification of thymol, a stabilizing agent present in halothane anaesthetic preparations. The method offers improvements in specificity and simplicity with respect to a current official procedure for thymol in halothane. Results for commercial preparations obtained by the proposed procedure demonstrate excellent precision and accuracy with RSD values for replicate analysis ranging from 0.11 to 0.74% and recoveries via fortification from 99.6 to 100.1%. The HPLC method was compared to compendial procedures for thymol bulk substance and halothane products. Chromatographic separation of other related phenolic preservatives used in pharmaceuticals suggests a more extensive application of the proposed procedure.

Keywords: *Thymol; halothane preparations; HPLC; phenolic preservatives.*

Introduction

Halothane (2-bromo-2-chloro-1,1,1-trifluoroethane) is a widely used inhalation anaesthetic having rapid onset and recovery. It is a volatile, non-flammable, non-explosive liquid which is subject to slow decomposition in the presence of light and, therefore, manufactured in amber coloured bottles. The stability of halothane against oxidation is maintained by the addition of thymol at a concentration of 0.01% (w/w). Although other stabilizing agents have been suggested over the years [1], thymol has remained the preservative of choice for this medicinal agent.

Thymol (5-methyl-2-(1-methylethyl)phenol) is a monoterpene alcohol derived from the essential oil of various species of thyme (e.g. *Thymus vulgaris* L.) or prepared by synthetic means. The primary uses of thymol for medicinal purposes has been a topical antifungal or antibacterial agent and also as a counterirritant in topical analgesic formulations.

The analysis of thymol as a pharmaceutical agent by titrimetry has been used for over 80 years [2] and is based on the work of Koppeschaar [3] developed for phenol employing bromination followed by iodimetry. A current official procedure for thymol in antiseptic preparations has been adapted from these early bromatimetric techniques [4]. A variety

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of other analytical techniques have been reported for the determination of thymol in pharmaceuticals including UV spectrophotometry [5], colorimetry [6–9], thin-layer chromatography [10] and gas–liquid chromatography (GLC) [11, 12]. Few methods have been described for the assay of thymol at concentration levels used in halothane preparations as a preservative agent. Two current compendial procedures are both colorimetric and lack specificity [13, 14]. The British Pharmacopoeia method is a semi-quantitative limit test originally designed for application to thymol present in the anthelmintic agent, tetrachloroethylene. The US Pharmacopoeia procedure for thymol in halothane is an adaptation of the Gibbs reaction [15] using 2,6-dibromoquinone chlorimide under basic conditions with the formation of an indophenol chromophore. This reaction is carried out following the evaporation of the halocarbon in basic media. A GLC method has been reported for the determination of thymol present in several halocarbon-based anaesthetics including halothane [16].

More recent methodology for the isolation and determination of thymol utilizes high-performance liquid chromatography (HPLC) which has been directed toward the characterization of essential oils [17–21] with particular emphasis on the separation of the isomers, thymol and carvacrol. The use of both reversed-phase and adsorption modes of HPLC have been investigated in these studies. This chromatographic technique offers specificity, sensitivity and accuracy with minimal sample preparation prior to analysis. A rapid procedure was sought having these features for the quantitative assessment of thymol stabilizer in halothane products for possible adaptation to regulatory work.

This communication describes a simple liquid chromatographic method for thymol which has been applied to the bulk substance and commercial halothane USP preparations containing this stabilizing agent.

Experimental

Reagents and chemicals

Methanol and acetonitrile — HPLC grade (Burdick and Jackson, Muskegon, MI, USA) and distilled, deionized water passed through a 0.22 μm filter (Versapor, Gelman Sciences, Ann Arbor, MI, USA) were used throughout the study. Thymol, Ph. Helv. grade (99.44% by titration) was employed as the reference standard (Fluka Chemical Corp., Ronkonkoma, NY, USA). Other reference materials were obtained from the following sources: 4-chlorothymol and carvacrol (Aldrich Chemical Co., Milwaukee, WI, USA), 4-chlorophenol and 4-chloro-3-methylphenol (Eastman Kodak Co., Rochester, NY, USA), phenol (Mallinckrodt Inc., Paris, KY, USA) and 4-chloro-3,5-xylenol (Ferro Corp., Bedford, OH, USA). Samples of thymol bulk material and halothane USP were obtained through various commercial sources including Halocarbon Laboratories Inc. (Hackensack, NJ, USA), Ayerst Laboratories Inc. (New York, NY, USA) and Abbott Laboratories (North Chicago, IL, USA).

HPLC instrumentation

The liquid chromatograph consisted of an Altex/Beckman 100A solvent delivery system (Altex/Beckman, Berkeley, CA, USA), a Rheodyne 7120 injection valve equipped with a 20.0 μl loop (Rheodyne Inc., Cotati, CA, USA), a Kratos 757 variable wavelength detector (ABI Analytical, Ramsey, NJ, USA) and a HP 3385A computing integrator (Hewlett–Packard, Palo Alto, CA, USA).

Chromatographic conditions

Separations were carried out with a 25 cm × 4.6 mm i.d. column containing 5 μm Ultrasphere Octyl packing (Beckman Instruments, Berkeley, CA, USA). The mobile phase was methanol–water (75:25%, v/v) prepared by diluting 750 ml of methanol to about 1 l with water, cooling to room temperature and adjusting to volume. The mixture was passed through a 47-mm dia., 0.45 μm porosity cellulose triacetate membrane filter (Gelman Sciences) under vacuum prior to use. The column flow rate was 1.0 ml min^{-1} under ambient conditions with a detection wavelength of 275 nm and a chart speed of 0.5 cm min^{-1}. Other columns used in the study for comparative purposes were: 10-μm Resolvex C-8, 25 cm × 4.6 mm i.d. (Fisher Scientific, Pittsburgh, PA, USA), 10-μm Lichrosorb RP-8, 25 cm × 4.6 mm i.d. (EM Science, Cherry Hill, NJ, USA), 5-μm Supelcosil LC-8 DB, 15 cm × 4.6 mm i.d. (Supelco Inc., Bellefonte, PA, USA) and 5–6 μm Zorbax C-8, 15 cm × 4.6 mm i.d. (DuPont Co., Wilmington, DE, USA).

Procedure

Bulk thymol material. An accurately weighed portion of the ground crystalline material equivalent to 100 mg was transferred to a 100-ml volumetric flask, and dissolved in and diluted to volume with mobile phase. A 1.0 ml aliquot of this solution was further diluted to 100.0 ml with the same solvent.

Halothane USP preparations. A 5.0-ml aliquot of the product was transferred to a 100-ml volumetric flask containing 25 ml mobile phase, the mixture swirled and diluted to volume with the same solvent.

Thymol reference standard. An accurately weighed portion of thymol reference material equivalent to 100.0 mg was transferred to a 100-ml volumetric flask, dissolved in and diluted with mobile phase. Further dilutions were made to a final thymol concentration of 0.01 mg ml^{-1}. Twenty microlitres of this solution was chromatographed under the conditions described above and the sensitivity adjusted to provide a peak response for thymol of 50–70% FSD.

Duplicate 20-μl volumes of the diluted sample preparation and thymol reference standard were chromatographed using a bracketing injection sequence and quantitation was achieved by comparison of the average peak areas obtained. The calculation for the halothane preparations was based on a w/w relationship and incorporated the specific gravity of the product.

Recovery study

Three samples of halothane USP drug product representing each commercial source were fortified at the 100% level in the following manner and carried through the procedure: a 5.0-ml aliquot was combined with 10.0 ml of a thymol standard solution having a concentration of 0.10 mg ml^{-1} in a 200-ml volumetric flask and diluted to volume with mobile phase. These determinations were performed simultaneously with the replicate analyses for each of the three products.

Separation of thymol and carvacrol

Several experiments were conducted employing variations in eluent composition to determine the retention behaviour of these isomers. Individual standard solutions of thymol and carvacrol and a mixture at a concentration of 0.02 mg ml^{-1} for each

compound were prepared in the appropriate mobile phase. Mobile phase mixtures consisting of methanol–water, acetonitrile–water and combinations of these three components were examined in order to resolve the two compounds. The mobile phase mixtures containing acetonitrile were passed through a 0.45-μm porosity nylon-66 membrane filter prior to use.

Results and Discussion

A mobile phase composition of methanol–water (75:25%, v/v) was determined to be optimum in regard to the resolution of thymol and a response associated with halothane. Methanol in water at this concentration was found to have superior solvation properties in the presence of halothane in comparison to acetonitrile in water mixtures of similar polarity (i.e. 65% acetonitrile in water) where two phases were observed. It was imperative that the mobile phase and the dilution solvent for the samples and reference standard be similar to provide optimal peak shape. The use of methanol alone as a diluent under the established mobile phase composition resulted in a distorted analyte peak having frontside skew. The optimal detection wavelength determined from the UV spectrum of a reference standard prepared in the mobile phase was 275 nm.

Comparative assay results

A comparison of assay results obtained by the USP XXI titration procedure [22] and by HPLC for pharmaceutical grade thymol is shown in Table 1. The products represented three commercial sources with the assay values by both methods within the compendial requirements for purity of 99.0–101.0%. The titrimetric procedure was found to be much more manipulative and time consuming than the chromatographic approach with an endpoint that was difficult to discern.

Table 1
Comparative assay results for thymol USP

	Percent purity*		
		HPLC procedure	
Product	USP XXI titration procedure	Mean (n = 3)	RSD (%)
1	99.11, 99.46	100.5	0.64
2	98.90, 99.02	100.1	0.47
3	99.26, 99.10	99.5	0.88

*USP XXI requirement: 99.0–101.0%.

Table 2 is a compilation of the assay results for thymol in commercial halothane obtained by the USP XXI colorimetric procedure and by HPLC. The samples included represent 12 different lots of product within three brands and were all labelled to contain 0.01% thymol (w/w). All products were found to be within the official requirements of 0.008–0.012% using either method. However, the comparative assay values shown indicate a systematic error having a positive bias (2.9–5.2%) with respect to the colorimetric procedure. Employing the method of standard additions [23], we have traced the possible source of this error to a matrix effect. The colorimetric procedure utilizes a reagent blank throughout since the use of a sample blank (i.e. unpreserved halothane) is not feasible. The re-assay of products 8 and 10 (from Table 2) using the

Table 2
Comparative assay results for thymol in commercial halothane USP labelled to contain 0.01% (w/w) thymol

Product	Source	Percent thymol* (w/w) × 10^2 USP XXI colorimetric procedure	HPLC procedure Mean (n = 3)	RSD (%)
1	A	1.050, 1.055	1.015	0.11
2	A	1.057, 1.060	1.013	0.59
3	B	1.055, 1.061	1.010	0.74
4	C	1.090, 1.092	1.062	0.52
5	C	1.036, 1.043	0.996	0.53
6	A	1.040, 1.047	1.000	0.61
7	A	1.036, 1.022	0.977	0.39
8	A	1.000, 1.001	0.950	0.56
9	B	1.067, 1.081	1.029	0.35
10	B	1.040, 1.044	1.003	0.32
11	B	1.063, 1.067	1.016	0.25
12	B	1.033, 1.037	0.994†	0.23

*USP XXI requirement: 0.008–0.012%.
†n = 6.

Table 3
Assay results for thymol from selected products of halothane USP using the USP colorimetric procedure and USP colorimetric procedure with standard additions

Product	Percent thymol (w/w) × 10^2 USP XXI colorimetric	USP XXI colorimetric — standard additions
8A	0.996, 1.000	0.949
10B	1.028, 1.042	1.003

USP XXI colorimetric procedure and the same procedure with standard additions provided the results shown in Table 3. The values obtained by the standard addition technique were lower in each case than the compendial procedure but consistent with those found by HPLC suggesting the presence of a matrix interference. Minor impurities present in halothane or conversion products formed during the sample evaporation step under basic conditions could have contributed to the slightly elevated absorbance values associated with the colorimetric procedure.

Typical chromatograms of a halothane sample dilution and thymol reference standard are shown in Fig. 1. The response observed at about 4.5 min for the sample is due to the halocarbon. This response was not altered by multiple extraction of the product with aqueous 0.5 N sodium hydroxide, whereas the peak area for thymol was reduced considerably in this process via the formation of the sodium salt of the phenoxide ion. No attempt was made to determine the halothane purity using HPLC since GLC is a more suitable and specific technique for this purpose [14, 24].

Precision and accuracy

The intra-laboratory precision (RSD) of the HPLC procedure based on triplicate assay values was well below 1.0% for all of the bulk material and anaesthetic preparation assays. The accuracy of the proposed HPLC procedure was assessed by adding known amounts of thymol to aliquots of product representing each of the commercial brands of halothane USP. Recovery values based on the addition of 1.00 mg thymol to products designated in Table 2 as 7A, 3B and 4C were 99.8, 100.1 and 99.6%, respectively.

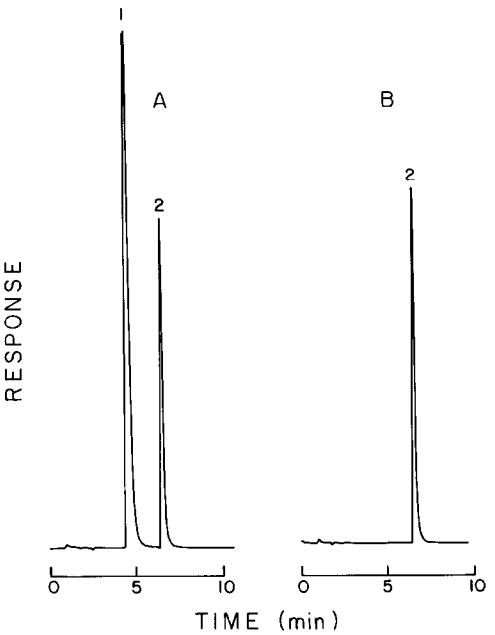

Figure 1
Chromatograms of commercial halothane USP containing thymol stabilizer (A) and thymol reference standard (B). 1, Halothane; 2, thymol. Conditions: column, Ultrasphere Octyl (5 μm) 25 cm × 4.6 mm i.d.; mobile phase, methanol–water (75:25%, v/v); flow rate, 1.0 ml min^{-1}; detection, 275 nm; temperature, ambient.

Chromatography — other phenolic compounds

Retention data for thymol and several related phenolic compounds used as preservatives or having anti-infective properties are shown in Table 4. A chromatogram of a reference mixture containing five of these compounds is shown in Fig. 2. This data suggest that the procedure might have application to other pharmaceuticals containing these related agents. Included with the retention data is that for carvacrol, a positional isomer of thymol. This pair of compounds was unresolved under the chromatographic conditions described for the assay with a retention for carvacrol relative to thymol of 0.98. Thymol obtained from natural sources would exhibit a small shoulder on the frontside of the thymol response due to carvacrol. A cursory examination of the literature indicated considerable interest in this isomeric pair as constituents of certain essential oils. We have resolved these two compounds employing a ternary mobile phase consisting of methanol–acetonitrile–water (30:25:45%, v/v). A representative chromatogram is provided as Fig. 3 with a resolution factor (R_s) calculated to be 1.95. This separation has also been reported using binary mobile phase conditions with reversed-phase [18, 19] and adsorption modes [21] of HPLC.

Column comparison

A brief study was performed to determine the chromatographic characteristics of different brands of octylsilane-type columns. Comparative data related to the retention and resolution between thymol and halothane are shown in Table 5 for five columns including the brand used (column 1) to develop the assay procedure. With the exception of column 2, all of the columns were found to be suitable for the assay of thymol by the proposed procedure. Column 2 exhibited significant peak tailing for thymol and failed to adequately resolve the five component mixture shown in Fig. 2.

Table 4
Retention data for thymol and several related phenolic compounds of pharmaceutical interest

Compound	Retention time* (min)	Capacity factor (k')
Phenol	3.58	0.28
4-Chlorophenol	4.36	0.56
4-Chloro-3-methylphenol	4.99	0.78
4-Chloro-3,5-xylenol	6.24	1.23
Carvacrol	6.67	1.38
Thymol	6.82	1.44
4-Chlorothymol	10.11	2.61

*Solvent front retention for 60% methanol in water (t_0) = 2.80 min.

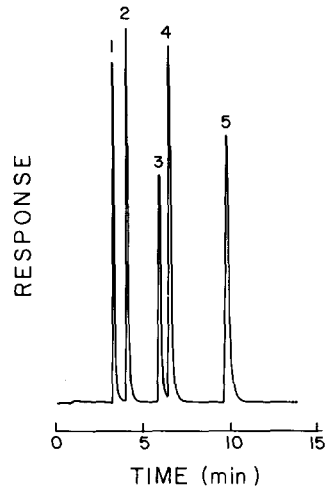

Figure 2
Chromatogram of a standard mixture of thymol and related phenols. 1, Phenol (0.1 μg); 2, 4-chlorophenol (0.1 μg); 3,4-chloro-3,5-xylenol (0.1 μg); 4, thymol (0.1 μg); 5, 4-chlorothymol (0.15 μg). Conditions as in Fig. 1.

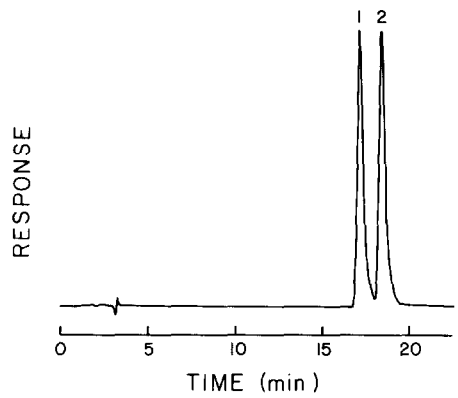

Figure 3
Chromatogram of a standard mixture of carvacrol and thymol. 1, Carvacrol (0.4 μg); 2, thymol (0.4 μg). Mobile phase: methanol–acetonitrile–water (30:25:45%, v/v). Other conditions as in Fig. 1.

Linearity and detection limit

Under the established chromatographic conditions, the analyte response was observed to be linear over at least a 50-fold range in concentration (0.001–0.050 mg ml^{-1}) with a correlation coefficient (r) of 0.9999. The minimum detectable quantity for thymol at a signal-to-noise ratio of 3:1 was 1 ng injected.

Table 5
Comparative retention and resolution data between thymol and halothane with various brands of octylsilane columns

Column*	Retention (min) Halothane	Thymol	Resolution (R_s)
(1) Ultrasphere Octyl	4.74	6.74	4.65
(2) Fisher Resolvex C-8	5.95	8.19	3.12
(3) Lichrosorb RP-8	6.42	9.10	4.47
(4) Supelcosil LC8-DB	3.14	4.65	4.72
(5) DuPont Zorbax C-8	3.67	5.76	5.47

*Column dimensions and packing particle size described under Experimental.

In conclusion, the proposed procedure for thymol is precise, accurate, simple to perform and overcomes several problems associated with the current USP XXI procedures for the bulk substance and halothane anaesthetic preparations. The method could find use for regulatory purposes and may have application to other phenolic ingredients in pharmaceuticals.

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