Journal of Chromatography, 568 (1991) 467–474
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam

CHROMBIO. 5934

Short Communication

Determination of trifluorothymidine in the eye using high-performance liquid chromatography

MARIS R. RIEGEL* and PHILIP P. ELLIS

Department of Ophthalmology, University of Colorado, School of Medicine, 4200 E. 9th Avenue, Denver, CO 80262 (USA)

(First received December 6th, 1990; revised manuscript received April 4th, 1991)

ABSTRACT

A high-performance liquid chromatographic assay for trifluorothymidine in biological matrices of the eye is presented. Sample pretreatment was based on liquid–liquid extraction with ethyl acetate. Following evaporation, the residue was analyzed using a reversed-phase octadecylsilane column with ultraviolet detection. The lower limit of detection is *ca.* 50 ng/ml (*ca.* 3 ng on column). The method is simple, reproducible and selective. Concentrations of trifluorothymidine in the aqueous humour, vitreous humour and cornea of normal rabbits after a 50-μl topical dose of 1% trifluorothymidine are reported.

INTRODUCTION

Trifluorothymidine (5-trifluoromethyl-2-deoxyuridine) or trifluoridine is an important antiviral agent for topical use in the treatment of deep and surface herpetic infections [1]. An animal study using a plaque inhibition assay technique and an *in vitro* corneal penetration study using radiolabeled drug have described the entry of trifluorothymidine into the eye [2,3]. Penetration of the drug has been visualized elegantly using secondary ion mass spectrometry [4]. High-performance liquid chromatographic (HPLC) assays presented in two clinical reports, however, reported the lowest detectable level of drug as 0.5 μg/ml [5,6]. Therefore, trifluorothymidine was not detected in the aqueous humour of patients with normal corneal epithelium and stroma, even after multiple instillations of the drug [5,6]. HPLC analysis of total vitreous following intravitreal administration of liposome-encapsulated trifluorothymidine has been reported; an internal standard was lacking and the reported sensitivity of the assay was 0.1 μg/ml [7].

The present paper describes a simple, isocratic method based on reversed-phase liquid chromatography and UV detection which is fast, reproducible and considerably more sensitive than the previously published assays for trifluorothymidine in ocular fluids. The lower limit of detection is *ca.* 0.05 μg/ml. The assay

0378-4347/91/$03.50

requires only the most basic equipment. A structurally similar internal standard is used and no prior protein precipitation is necessary. The method is sufficiently sensitive to measure trifluorothymidine concentrations achieved in individual aqueous humour samples from rabbits with healthy corneal epithelium following a single topical administration of the drug. The assay also is appplicable to aliquots of vitreous humour of the eye and to the cornea. The technique was developed to compare different dose delivery systems.

EXPERIMENTAL

Chemicals

Trifluorothymidine and other nucleosides and nucleotides were obtained from Sigma (St. Louis, MO, USA). 3-Methylthymidine was obtained from Aldrich (Milwaukee, WI, USA). A commercially available solution of 1% trifluorothymidine (Viroptic, Burroughs Wellcome, Research Park Triangle, NC, USA) was used for administration to laboratory animals. Commercial preparations of therapeutic agents tested for drug interference were obtained from the University Hospital Pharmacy. Chromatography solvents, distilled-in-glass grade, were purchased from Burdick & Jackson Labs. (Muskegon, MI, USA). All other chemicals were reagent grade. Water was double-distilled. Solutions were filtered through a 0.45-μm Millipore filter prior to use.

Chromatography

The Beckman (Anaheim, CA, USA) Model 330 isocratic system consisting of a Model 110 A pump, Model 210 sample injection valve with a 20-μl injection loop and Model 153 detector for UV detection at 254 nm was used in conjunction with a Hewlett-Packard (Hewlett-Packard, Avondale, PA, USA) 3390 integrator. An Altex-Beckman Ultrasphere reversed-phase octadecylsilane (RP-ODS) column, particle size 5 μm, 25 cm x 4.5 mm I.D. was used. The mobile phase was 0.01 *M* acetate buffer (pH 3.88)–acetonitrile (87.5:12.5, v/v) with 10^{-3} *M* sodium hexanesulfonate. The mobile phase was pumped at 1.3 ml/min at ambient temperature.

Assay procedure

A 100-μl aliquot of the standard solution or fluid sample was placed in a 15-ml conical test tube together with 75 μl of 0.2 *M* sodium acetate buffer (pH 3.88). Corneal buttons were trimmed free of sclera, blotted gently on a filter paper, minced and placed in a tared conical tube; tubes were reweighed and buffer was added as above. A 25-μl volume of the internal standard 3-methylthymidine (8.2 μg/ml in water) was added and the mixture was mixed immediately. The sample was extracted twice by swirl-mixing for 90 s with 1.5 ml of ethyl acetate. The layers were separated by centrifugation (5 min; 300 *g*), the organic layers combined and dried under nitrogen. The residue was dissolved in mobile phase and injected into the HPLC system.

Standard curve, precision and recovery studies

Standard curves were constructed by analyzing samples of aqueous or vitreous humour containing known amounts of trifluorothymidine in a concentration range of 0.05–32.0 μg/ml. Corneal buttons were spiked with trifluorothymidine to yield concentrations of 0.05, 0.08, 0.16, 0.31 and 0.62 μg per cornea. The correlation coefficient and the regression equations were calculated using least-squares linear regression analysis by correlating the peak height against the corresponding spiked concentrations. Within-day variability was determined by analyzing ten replicate samples of aqueous and vitreous humour containing trifluorothymidine at a concentration of 1 μg/ml. Aqueous humour and vitreous humour samples were analyzed daily for ten days to determine between-day variability. Recovery of the drug was calculated by comparing aqueous standard solutions (no extraction), with extracted spiked rabbit aqueous humour, vitreous humour or cornea at the same concentrations.

TABLE I

AGENTS TESTED FOR INTERFERENCE IN THE CHROMATOGRAPHY OF TRIFLUOROTHYMIDINE (t_R 5.1 min) AND METHYLTHYMIDINE (t_R 4.1 min)

Non-interfering compound	Interfering compounds
Antazoline phosphate	Sulfacetamide sodium (t_R 5.0 min)
Atropine sulfate	
Bacitracin[a] (t_R 2.8 min)	
Chloramphenicol (t_R 43.1 min)	
Cyclopentolate hydrochloride	
Dexamethasone	
Dipivefrin hydrochloride	
Epinephrine	
Epinephryl borate	
Erythromycin	
Fluorometholone	
Flurbiprofen sodium (t_R 2.32 min)	
Homatropine	
Hydrocortisone acetate	
Naphazoline hydrochloride	
Neomycin sulfate	
Phospholine iodide	
Polymixin B sulfate[a]	
Procaine	
Proparacaine hydrochloride	
Scopolamine hydrobromide (t_R 31.55 min)	
Sulfamethoxazole (t_R 31.4 min)	
Tetracycline hydrochloride	
Timolol maleate	
Tropicamide	

[a] Tested at 100U.

Selectivity and interference studies

To identify potential interference by endogenous components, samples of rabbit aqueous humour, vitreous humour and minced corneal button obtained from animals not treated with trifluorothymidine were analyzed without the addition of internal standard. All samples were assayed again after freezing at −20°C; cornea samples were frozen in 75 μl of assay buffer. Aliquots of aqueous humour and vitreous humour from rabbits receiving a topical dose of trifluorothymidine were assayed both immediately after collection and weekly for one month after freezing at −20°C to assess stability.

Interference by therapeutic agents frequently used with trifluorothymidine (Table I) was evaluated. Some commercially available purine and pyrimidine

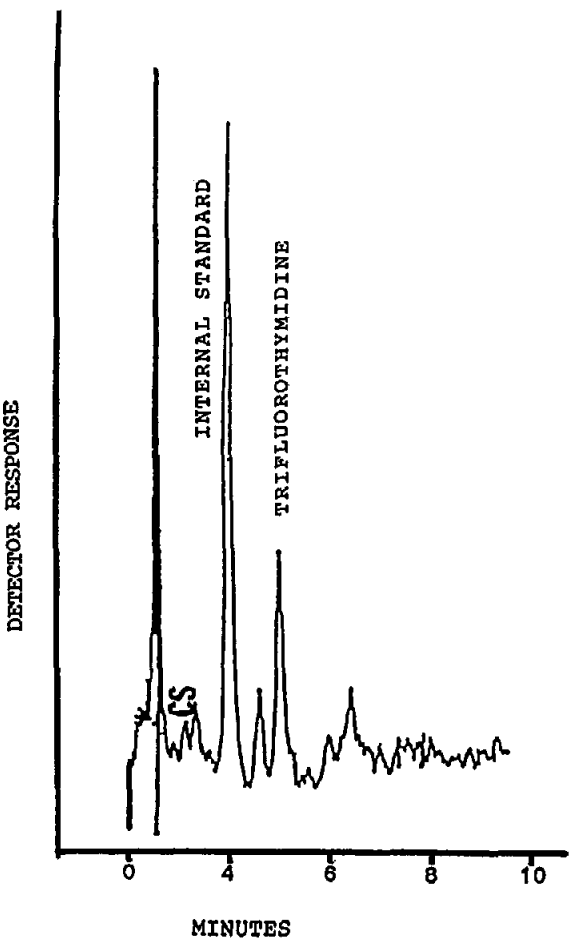

Fig. 1. Chromatogram of the standards carried through the extraction procedure: trifluorothymidine, (t_R 5.1 min) and methylthymidine (t_R 4.1 min). Concentration: 0.36 μg/ml.

nucleosides and nucleotides were analyzed to verify the retention times of naturally occurring compounds. Several dideoxynucleosides under investigation for use in the treatment of immunosuppressive disease also were tested. All compounds were tested for interference at a concentration of 10 μg/ml.

Animal studies

New Zealand white rabbits (Bell Rabbitry, Clovis, NM, USA) weighing approximately 2.5–3 kg were given a 50-μl dose of 1% trifluorothymidine topically in one eye. The contralateral eye served as a control. After 30 min, the rabbits were sacrificed and aqueous humour, vitreous humour and cornea were removed and assayed as described.

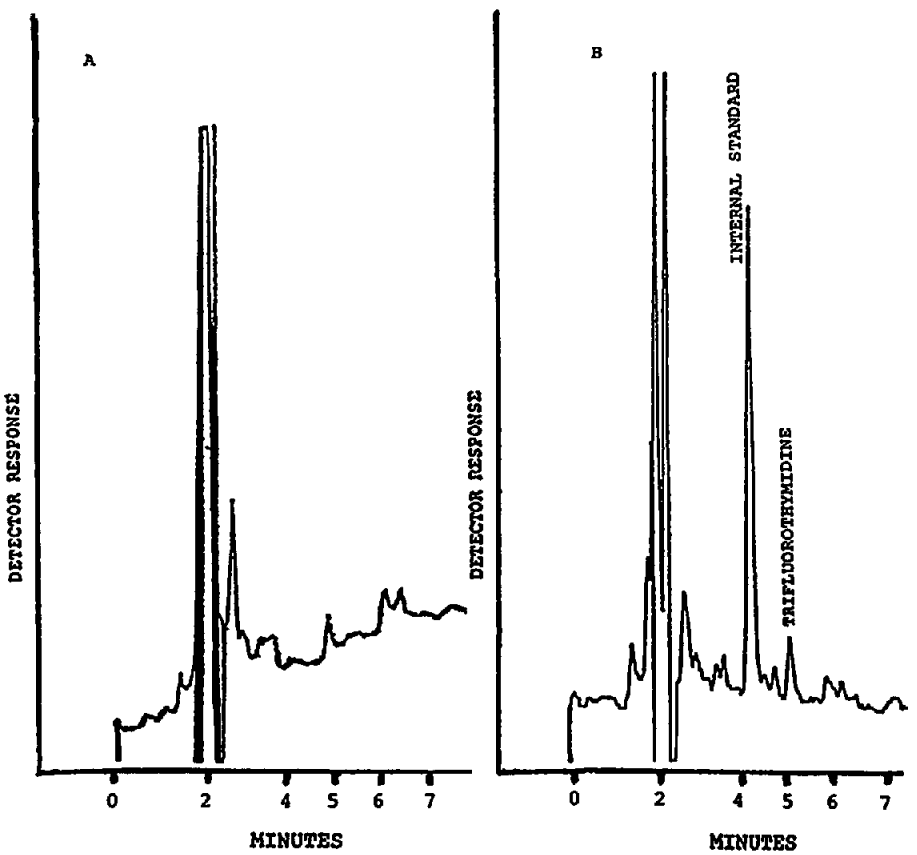

Fig. 2. Chromatograms obtained by analysis of rabbit aqueous humour (A) obtained from animals not treated with trifluorothymidine and without addition of internal standard and (B) obtained from a rabbit 30 min after topical administration of a 50-μl dose of 1% trifluorothymidine. Concentration: 0.28 μg/ml.

RESULTS AND DISCUSSION

Under the chromatographic conditions described the retention times of trifluorothymidine and the internal standard were 5.1 and 4.1 min, respectively (Fig. 1). Analysis of a series of aqueous humour, vitreous humour and cornea samples containing known amounts of trifluorothymidine yielded a standard curve in which the concentration of the drug was linearly related to the trifluorothymidine: internal standard peak-height ratios. The data fit the equation of a straight line: peak-height ratio = $0.9708x + 0.0360$. Least-squares analysis yielded a coefficient of correlation (r) of 0.9999. The lower limit of detection is *ca.* 3 ng on the column. The within-day analysis of aqueous humour samples ($n = 10$) containing 1 μg/ml trifluorothymidine gave a mean drug concentration of 0.98 μg/ml with a coefficient of variation (C.V.) of 5.25% while between-day results were

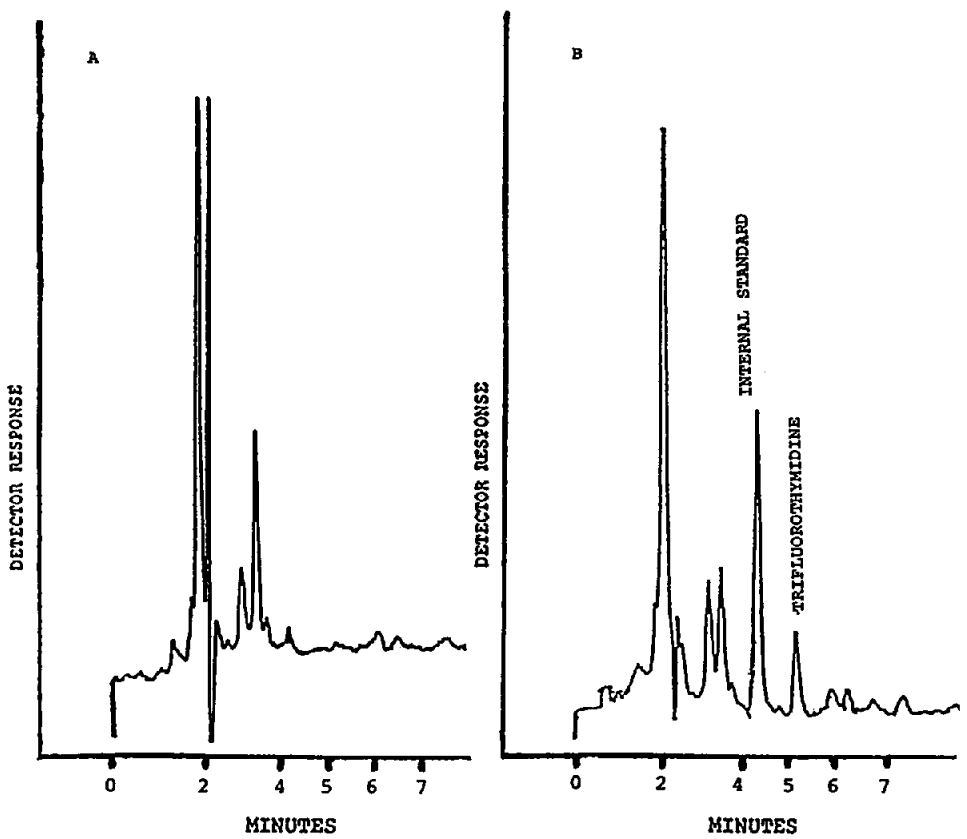

Fig. 3. Chromatograms obtained by analysis of rabbit vitreous humour (A) obtained from animals not treated with trifluorothymidine and without addition of internal standard and (B) obtained from a rabbit 30 min after topical administration of a 50-μl dose of 1% trifluorothymidine. Concentration = 0.24 μg/ml.

0.97 μg/ml (C.V. 4.8%). The within-day and between-day results following analysis of vitreous humour samples (n = 10) were 0.96 μg/ml (C.V. 5%) and 0.98 μg/ml (C.V. 4.8%), respectively. The C.V. for analysis of cornea was not determined due to limited availability of corneal material.

Recovery of drug (mean ± S.D.) from aqueous or vitreous humour was 99.4 ± 2% (n = 12) and from fresh cornea, 92.3 ± 7.1% (n = 6). Curves constructed from trifluorothymidine extracted from water were essentially identical to those from the biological matrices. No interference by endogenous compounds was found when aqueous humour (Fig. 2A), vitreous humour (Fig. 3A) or fresh cornea (Fig. 4A) from untreated rabbits were analyzed. In Fig. 4A the extracted sample is diluted with only 50 μl of mobile phase. Although 1-h chromatograms of blank aqueous, vitreous and fresh cornea samples exhibited no peaks after 7

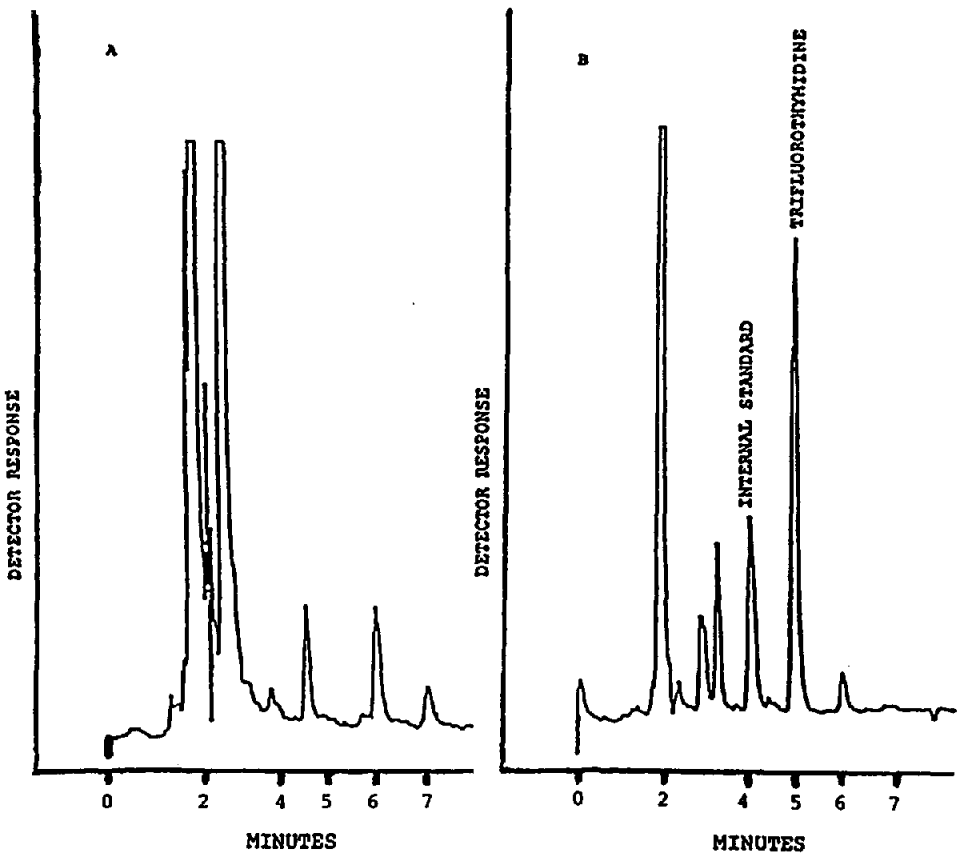

Fig. 4. Chromatograms obtained by analysis of fresh rabbit cornea (A) obtained from animals not treated with trifluorothymidine and without addition of internal standard and (B) obtained from a rabbit 30 min after topical administration of a 50-μl dose of 1% trifluorothymidine. Concentration: 0.25 μg; 6.1 μg/g of cornea.

min, chromatograms of cornea that had been frozen before assaying displayed interfering peaks and numerous peaks with longer retention times. Aqueous and vitreous humour from rabbits receiving topical trifluorothymidine was stable for at least two weeks frozen at −20°C. Of the agents tested in Table I, only sulfacetamide sodium, retention time (t_R) 5.0 min, interfered. More polar nucleotides and nucleosides eluted with or near the solvent front (data not shown) while more lipophilic nucleosides, *e.g.* 2′,3′-dideoxycytidine (t_R 4.47 min) 3′-deoxythymidine (t_R 3.6 min) and 5′-deoxythymidine (t_R 4.73 min), eluted near the trifluorothymidine or internal standard peaks. They did not interfere when tested in the presence of the standards. Figs. 2B, 3B and 4B are chromatograms of aqueous humour, vitreous humour and cornea from a rabbit treated with a single instillation of trifluorothymidine.

CONCLUSIONS

To our knowledge, this is the only HPLC assay of trifluorothymidine available for studying drug penetration into the aqueous humour, vitreous humour and cornea following a single topical dose of the drug. The method described here is simple, reproducible, selective and more sensitive than previously published aqueous humour assays. The assay is applicable to evaluation of drug administration techniques and other research applications.

ACKNOWLEDGEMENT

This work was supported by a grant from Research to Prevent Blindness, Inc.

REFERENCES

1 A. A. Carmine, R. N. Brogden, R. C. Heel, T. M. Speight and G. S. Avery, *Drugs*, 23 (1982) 329.
2 W. J. O'Brien and H. F. Edelhauser, *Invest. Ophthalmol. Vis. Sci.*, 16 (1977) 1903.
3 J. Sugar, E. Varnell, Y. Centifanto and H. E. Kaufman, *Invest. Ophthalmol. Vis. Sci.*, 12 (1973) 532.
4 J. M. Legeais, P. Hallegot, J. Chabala, G. Renard, R. Levi-Setti and P. Galle, *Curr. Eye Res.*, 8 (1989) 971.
5 R. H. Poirier, J. D. Kingham, P. deMiranda and M. Annel, *Arch. Ophthalmol.*, 100 (1982) 1964.
6 D. Pavan-Langston and D. J. Nelson, *Am. J. Ophthalmol.*, 87 (1979) 814.
7 K.-R. Liu, G. A. Peyman, B. Khoobehi, H. Alkan and R. Fiscella, *Ophthalmology*, 94 (1987) 1155.