Journal of Chromatography, 505 (1990) 349–356
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROM. 22 293

Determination of tebufelone (a new anti-inflammatory drug) strength and stability in bulk drug, dosage formulations and feed admixtures by reversed-phase high-performance liquid chromatography

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(First received June 27th, 1989; revised manuscript received January 22nd, 1990)

SUMMARY

A rugged reversed-phase high-performance liquid chromatographic method suitable for the quantitative determination of tebufelone, a new anti-inflammatory drug, in bulk drug, various pharmaceutical formulations and animal feed admixtures is described. Tebufelone was easily separated from synthetic by-products and detected by ultraviolet absorption (280 nm). Standard curves were linear ($r^2 > 0.999$) over 2 orders of magnitude with a detection limit of 0.1 μg/ml at a signal-to-noise ratio of 2 (0.05 ml injected). Recovery of tebufelone from bulk drug and dosage formulataions was > 99% with a coefficient of variation of 1.8% throughout the range of the standard curve. Recovery of tebufelone from feed admixtures was 96–102% with a < 5% relative standard deviation at the levels assayed.

INTRODUCTION

Tebufelone {NE-11740: 1-[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]-5-hexyn-1-one} is a new, highly potent anti-inflammatory drug which has exhibited efficacy in a variety of animal models[1]. The compound (Fig. 1) is a member of the di-*tert*.-butylphenol (DTBP) class of non-steroidal anti-inflammatory drugs (NSAIDs). Unlike conventional NSAIDs (*e.g.* indomethacin), which only block cyclooxygenase (CO) activity, tebufelone is an inhibitor of both CO and 5-lipoxygenase (LO) enzymes[2,3]. Some products of CO and LO activity, prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4) in particular, have been shown to mediate the pain, edema and tissue destruction associated with inflammatory processes[4,5].

Several DTBP anti-inflammatory agents[6–9] have been developed in recent years and it is hoped this new class of dual CO/LO inhibitory NSAIDs will prove advantageous for the treatment of arthritic, psoriatic or asthmatic conditions. Clinical evaluation of the therapeutic potential of drug candidates requires the development of

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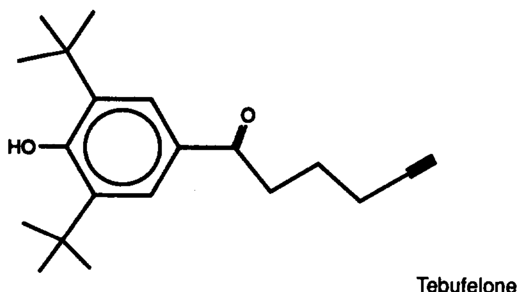
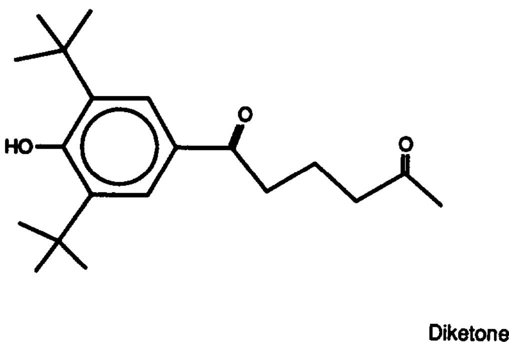
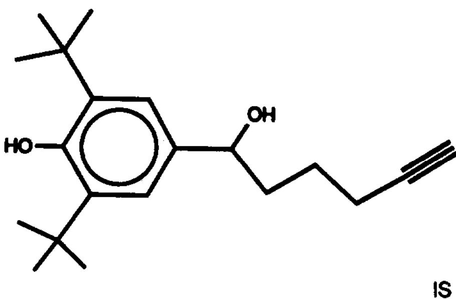


Tebufelone



Diketone



IS

Fig. 1. Chemical structure of tebufelone, the diketone synthetic by-product and the internal standard (IS).

analytical methods suitable for the measurement of active strength, purity and stability in bulk drug, dosage formulations and animal feed admixtures. Reversed-phase (RP) high-performance liquid chromatographic (HPLC) techniques have been successfully employed for the separation and quantification of a variety of phenolic analytes[10,11]. Verhagen *et al.*[12] have recently reported the development of a RP-HPLC procedure for the determination of butylated hydroxytoluene in bovine plasma. In addition, the effect of phenolic ring substitution on column (C_{18}) selectivity has also been investigated[13].

When performing chronic drug safety testing in animals, the preferred method of drug administration is via the diet *ad libitum* primarily for convenience sake. Consistently obtaining homogeneous drug-feed blends is a major concern during the

conduct of these types of studies[14]. The complex nature of animal feed matrices has necessitated the development of a chromatographic procedure utilizing an internal standard (IS). Butylated hydroxytoluene (BHT = 2,6-di-*tert.*-butyl-4-methylphenol) is structurally similar to tebufelone and normally would represent an ideal choice as an internal standard for quantitative purposes. Further, chromatographic methods for the determination of BHT in poultry premix and mixed feeds have recently appeared in the literature[15,16]. However, BHT is commonly added to animal feed as an antioxidant which precludes its use as a universal internal standard for animal feed analyses. For this reason, we chose to use a structural analogue of tebufelone (Fig. 1), in which the aliphatic carbonyl group has been reduced to a hydroxyl moeity, as an IS. The molar extinction coefficient (at 280 nm) for the IS is approximately 10 times less than that for tebufelone.

We report here the development of a facile RP-HPLC method for the determination of tebufelone in bulk drug preparations as well as dosage formulations. In addition, the method was modified to include an internal standard and methanolic extraction step for the determination of tebufelone in rodent chow. Separation is achieved isocratically using an octadecyl stationary phase and the drug is quantified via ultraviolet detection (280 nm).

EXPERIMENTAL

Reagents and chemicals

tebufelone reference standards, IS and ^{14}C-labeled tebufelone (48.2 μCi/mg) were synhesized at Miami Valley Labs. Reference standards were shown to be >99.9% pure by thermogravimetric and titrimetric methods of analysis. tebufelone drug product (tebufelone–pluronic F-108–polyethylene glycol 3350 in No. 00 hard gelatin capsules) was also manufactured at Miami Valley Labs. Capsules were prepared containing between 50 and 200 mg of drug active. Certified rodent chow meal No. 5002 from Ralston Purina (St. Louis, MO, U.S.A.) was formulated with tebufelone (1%) by the wet granulation method (ethanol slurry). Acetonitrile (HPLC grade) and potassium phosphate (ACS grade) were obtained from Fisher Scientific (Cincinnati, OH, U.S.A.). Phosphoric acid was purchased from EM Science (Hawthorne, NY, U.S.A.) and water was Milli-Q Reagent water (> 17 Ω resistivity) from Millipore (Bedford, MA, U.S.A.).

Procedure

Mobile phase preparation. A ternary mobile phase comprised of acetonitrile–water–potasium phosphate (70:30:0.01%) was prepared by adding 100 mg of potassium phosphate to *ca.* 250 ml of Milli-Q water. The pH of this solution was adjusted to 3.5 using phosphoric acid. This buffer solution was diluted to 300 ml with deionized water, added to 700 ml acetonitrile and degassed by aspiration for 10 min.

Standard and sample preparation for bulk drug and dosage formulations. Typically, four tebufelone standard solutions (20, 10, 4 and 2 μg/ml) were prepared in mobile phase. Bulk drug (usually 10 mg of raw material) was placed in a 100-ml volumetric flask and diluted to volume with mobile phase. A 1.0-ml aliquot of this stock solution was further diluted to 25 ml with mobile phase. Drug product was prepared by emptying contents of the capsules into a 250-ml volumetric flask through

a funnel. An accurate weight was obtained and sample was diluted to volume with mobile phase. A 1.0-ml aliquot of this stock solution was further diluted to 100 ml with mobile phase. Standard and sample diluent was injected directly (50 μl) onto the chromatograph. All analyses were performed in duplicate.

Standard preparation for IS-based method. Stock solutions of tebufelone and IS were prepared in mobile phase. Calibration standards, containing both components, were pepared in mobile phase from the stock solutions to yield final concentrations of: 1, 5, 10, 20, 35, 50 μg/ml tebufelone with 50 μg/ml IS in each. Also, an IS stock solution in ethanol was prepared at a concentration of 5 mg/ml.

Sample preparation for animal feed admixtures. Internal standard (100 μl of 5 mg/ml stock solution) was added to a 100-mg portion of the chow admixture in a 120 $\times$ 15 mm glass centrifuge tube (Fisher Scientific) followed by 10 ml of methanol as an extractant phase. This sample was mixed well for *ca.* 10 min to ensure complete extraction of tebufelone and IS. Centrifugation (2000 *g* for 10 min) was used to separate the solids from the organic phase, then 50 μl of the methanol phase was directly injected into the chromatograph.

Equipment and assay conditions

A modular system was employed which consisted of a Constametric III solvent delivery system (LDC/Milton Roy, Riviera Beach, FL, U.S.A.), a Wisp autosampler (Waters, Milford, MA, U.S.A.), and a Spectromonitor III variable-wavelength UV detector (LDC/Milton Roy). A 5-μm 25 cm $\times$ 4.6 mm I.D., Zorbax ODS analytical column (DuPont, Wilmington, DE, U.S.A.) was coupled with a 5-μm 30 mm $\times$ 4.6 mm I.D., RP-18 Brownlee pre-column (Rainin, Woburn, MA, U.S.A.). The analytes were eluted from the column isocratically using a mobile phase flow-rate of 2.0 ml/min and detection was achieved by monitoring the effluent at 280 nm.

Data were collected using version 4.1 of a chromatographic software package (Beckman, Fullerton, CA, U.S.A.) and a Mark 5 digimitry unit (Beckman) to digitize the signal from the detector. Data were then stored on an HP1000E data system (Hewlett-Packard, Avondale, PA, U.S.A.). A Model DU-50 spectrophotometer (Beckman) was used to obtain the UV–VIS spectum. Radioactive fractions were counted using a Packard Model 2000CA Scintillation counter (Downers Grove, IL, U.S.A.).

RESULTS AND DISCUSSION

Spectroscopic characterization of analyte

The UV–VIS absorption spectrum (200–400 nm) of tebufelone appears in Fig. 2. The spectrum is generally characteristic of phenolic compounds with a major band centered at 280 nm and high absorbance occurring below 240 nm[10]. The molar extinction coefficient (at 280 nm) was determined to be 5250 au/$M \cdot$ cm for tebufelone dissolved in acetonitrile–water–potassium phosphate (70:30:0.01). All excipients (pluronic F-108, polyethylene glycol 3350) used to formulate tebufelone drug product exhibited minimal absorption (essentially transparent in mobile phase) at 280 nm. Hence, the chromatographic effluent was monitored at 280 nm for tebufelone quantitative purposes.

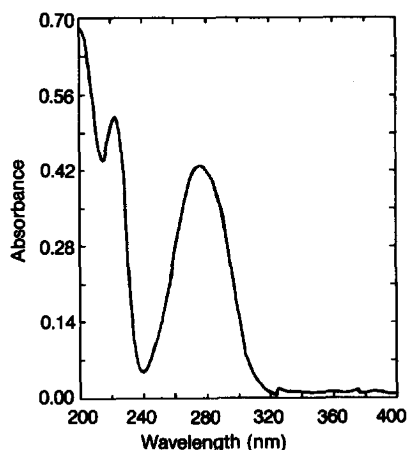


Fig. 2. UV–VIS absorption spectrum (200–400 nm) of a 10 μg/ml tebufelone in acetonitrile–water–potassium phosphate (70:30:0.01) solution.

Determination of tebufelone in bulk drug and dosage formulations

Chromatographically, tebufelone elutes with a retention time (t_R) of 5.6 min [capacity factor (k') = 3.5] under the assay conditions described above. As shown in Fig. 3, tebufelone is well resolved [resolution (R_s) > 2.0] from the diketone analogue which represents the primary synthesis by-product. This by-product is easily separated from active drug as a consequence of the synthetic purification sequence and not normally observed in final bulk drug preparations. During the course of routine analyses, no other potentially interfering peaks have been observed. Column efficiency (N) exceeded 12 000 plates and sample throughput can be maximized by employing shorter columns containing the 5-μm Zorbax C_{18} stationary phase without sacrificing resolution.

tebufelone standard curves (peak area) were linear between 0.2 and 20 μg/ml with a detection limit [signal-to-noise ratio (S/N) = 2] of 0.10 μg/ml. When standard

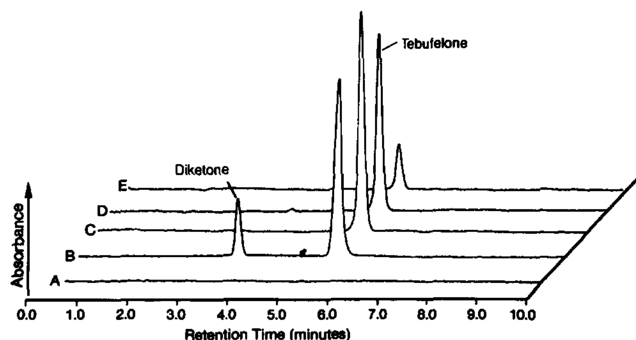


Fig. 3. Stacked plot of chromatograms including: (A) placebo capsule, (B) 4:1 mixture of tebufelone and diketone analogue, (C) 10 μg/ml tebufelone standard, (D) contents of a 200-mg capsule diluted to 8 μg/ml with mobile phase and (E) 2.0 μg/ml tebufelone standard. Detection was by UV absorption at 280 nm (50 μl injected).

data points are fitted using a first order linear regression algorithm, an intercept of 6.53 and slope of 150 was calculated. The correlation coefficient (r^2) for the analytical curve was always > 0.999. The change in response factor (R_f), for successive injections ($n = 10$) of a given analytical sample (Table I) was ± 1.8% relative standard deviation (R.S.D.) and retention times were precise to within ± 0.4% R.S.D. Recovery of [^{14}C]tebufelone injected on column, at concentrations bracketing the standard curve, was quantitative (100%). The accuracy of the method was confirmed by measuring recovery of tebufelone from spiked samples (Table II). Recovery of tebufelone added to excipient mixtures at the 1.9 and 11 μg/mg level was 101 ± 0.1% and 99 ± 0.7%, respectively. These measurements were precise to within 1% of the mean tebufelone concentration for each mixture.

Also, we have employed this methodology to monitor the stability of tebufelone bulk drug and tebufelone drug product (50- and 200-mg capsules). There has been no evidence of tebufelone degradation in samples stored under thermally accelerated conditions (23, 37, 49°C) with an atmospheric headspace over a one-year period. It is anticipated that pharmaceutical products containing tebufelone will be highly shelf-stable.

Determination of tebufelone in animal feed admixtures

As shown in Fig. 4, tebufelone is well resolved ($R_s > 2.0$) from the internal standard (IS, $t_R = 3.5$ min). There was no evidence of interfering chow matrix components within the tebufelone or IS retention windows. Calibration curves were linear over 1.5 orders of magnitude with a correlation coefficient (r^2) of 0.999. The limit of detection ($S/N = 2$) was determined to be 10 μg/g (5 ng on column) without preconcentration of the organic phase. The limit of detection for the method is restricted by the UV (280 nm) properties of the IS.

Instrumental precision was determined by calculating the variance in response factor (R_f = peak area ratio/tebufelone concentration) for a series ($n = 10$) of

TABLE I

HPLC SYSTEM PRECISION

The contents of a 200 mg tebufelone capsule were diluted in mobile phase and the resulting solution was analyzed repetitively ($n = 10$) to determine retention times (t_R) and response factor (R_f) reproducibility.

t_R (min)	R_f
5.45	19.79
5.46	19.41
5.46	19.89
5.47	19.62
5.47	19.51
5.47	19.61
5.43	20.30
5.43	20.44
5.43	19.62
5.41	19.49
Mean 5.45 ± 0.02	19.77 ± 0.35
R.S.D. 0.4%	1.8%

TABLE II

RECOVERY OF TEBUFELONE FROM SPIKED SAMPLES

Known quantities of tebufelone were added to pluronic F-108–polyethylene glycol 3350 (50:50) mixtures and analyzed by isocratic reversed-phase HPLC.

Sample No.	*Estimated concentration (μg/mg)*	*Determined concentration (μg/mg)*		*Recovery (%)*
1	1.93	1.95		101.2
2	1.93	1.95		101.2
3	1.93	1.96		101.4
			Mean	101.3 ± 0.1
			R.S.D.	0.1%
4	11.03	10.85		98.3
5	11.03	10.96		99.3
6	11.03	10.99		99.7
			Mean	99.1 ± 0.7
			R.S.D.	0.6%

calibration standards ranging in tebufelone concentration between 100 μg/g and 5 mg/g. Precision of tebufelone and IS measurements was 2.1% R.S.D. of the mean R_f (2.0 ± 0.04). Retention time reproducibility was quite acceptable at less than 0.4% coefficient of variation (C.V.) for both tebufelone (average t_R = 5.66 ± 0.02) and IS (average t_R = 3.52 ± 0.01).

Absolute recovery of tebufelone from rodent chow was determined to be 96–99% (0.08 to 2.7 mg/g range) using [^{14}C]tebufelone as a tracer. The use of a structural analogue of tebufelone as an internal standard precludes the need to correct for analyte losses subsequent to sample handling. The accuracy of the method was demonstrated by analyzing blank chow samples spiked with 0.1 mg/g (n=5) and 5.0 mg/g (n=3) tebufelone. Mean recoveries were 96.2% and 101.9%, respectively with an assay precision of between 1.5% and 5.0%. This methodology is rugged and

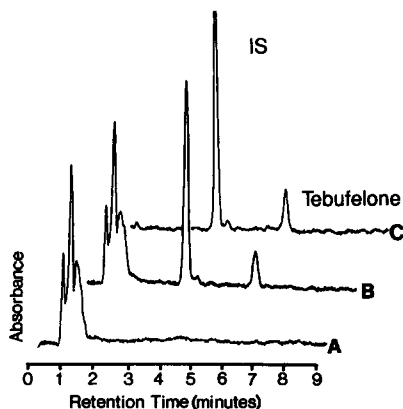


Fig. 4. Reversed-phase HPLC chromatograms of: (A) extract of 100 mg rodent chow, (B) extract of 100 mg rodent chow containing tebufelone (10 μg) and (C) a tebufelone–IS (1:50 ratio) calibration standard.

presently being employed to monitor the stability of tebufelone in rodent feed admixtures. It also may be broadly applicable for the determination of anti-inflammatory di-*tert.*-butylphenol analogues of pharmaceutical interest.

ACKNOWLEDGEMENTS

The authors wish to acknowledge the efforts of Ms. Susanne Menkedick and Mr. Edmund M. Blanken related to the conduct of the drug stability studies. Also the efforts of Messrs. Douglas J. Dobrozsi, Gary R. Kelm and Clarence Dixon, relating to the preparation of the drug-chow admixtures, are greatly appreciated.

REFERENCES

1 M. E. Loomans, J. A. Miller, R. S. Matthews, R. W. Farmer, D. A. Lade, R. A. Underwood, K. L. Skare and H. H. Tai, *Fourth Int. Conf. Inflamm. Res. Assoc., White Haven, PA, October 23–27, 1988*, Abstract No. 51.
2 S. M. Weisman, B. A. Hynd, M. J. Doyle, C. W. Coggeshall, R. M. Kaffenberger, T. H. Eichhold, D. L. Holloway and K. R. Wehmeyer, *Fourth Int. Conf. Inflam. Res. Assoc., White Haven, PA, October 23–27, 1988*, Abstract No. 66.
3 M. J. Doyle, T. H. Eichhold, B. A. Hynd and S. M. Weisman, *J. Pharm. Biomed. Anal.*, (1990) in press.
4 J. Vane and R. Botting, *FASEB J.*, 1 (1987) 89.
5 M. A. Bray, *Agents Actions*, 19 (1/2), (1986) 87.
6 E. S. Lazer, H. C. Wong, G. J. Possanza, A. G. Graham and P. R. Farina, *J. Med. Chem.*, 32 (1989) 100.
7 G. G. I. Moore and K. F. Swingle, *Agents Actions*, 12 (1982) 674.
8 H. Ikuta, H. Shirota, Y. Kobayashi, K. Yamagishi, I. Yamanda, I. Yamatsu and K. Katayama, *J. Med. Chem.*, 30 (1987) 1955.
9 T. Hidaka, K. Hosoe, Y. Ariki, K. Takeo, T. Yamashiti, I. Katsumi, H. Kondo, K. Yamashiti and K. Watanabe, *Jpn. J. Pharmacol*, 36 (1984) 77.
10 E. Tesarova and V. Pacakova, *Chromatographia*, 17 (1983) 269.
11 K. Robards and S. Dilli, *Analyst (London)*, 112 (1987) 933.
12 H. Verhagen, H. H. W. Thijssen and J. C. S. Kleinjans, *J. Chromatogr.*, 422 (1987) 288.
13 E. Burtscher, H. Binder, R. Concin and O. Bobleter, *J. Chromatogr.*, 252 (1982) 167.
14 G. O. Kuhn, J. J. Rollheiser, B. A. Schworer and C. W. Jameson, in D. B. Walters and C. W. Jameson, (Editors), *Chemistry for Toxicity Testing*, Butterworth, Boston, MA, 1984, Ch. 5, p. 59.
15 D. Beker and V. Lovrec, *J. Chromatogr.*, 393 (1987) 459.
16 R. Frankel and I. Slad, *Fresenius' Z. Anal. Chem.*, 331 (7) (1988) 760.