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REVERSED-PHASE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC ASSAY FOR THE DETERMINATION OF POTENCY AND IMPURITIES IN TAZADOLENE SUCCINATE BULK DRUG AND CAPSULES

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SUMMARY

A reversed-phase assay based on high-performance liquid chromatography with a water–acetonitrile–tetrahydrofuran (THF)–triethylamine (TEA)–perchloric acid (pH 2.5) mobile phase and a Zorbax C_8 column has been validated for the determination of the purity of tazadolene succinate (I) [*E*-(±)-1-(2-benzylidenecyclohexyl)azetidine succinate, U-53996H] bulk drug, the potency of tazadolene succinate hard-filled capsule formulations and impurity levels in bulk drug. The system resolves *E*- and *Z*-isomers and other structurally related molecules. Retention of these compounds is mainly dependent on the amount of acetonitrile and THF in the mobile phase. An amine must be present in the mobile phase to bring about elution of I. The potency assay utilizes testosterone as internal standard. Potency assays exhibited relative standard deviations (R.S.D.) of less than 1%. Quantitative recovery from hard-filled capsules (HFC) is obtained by using a simple extraction procedure. Potential process impurities, potential degradation products, and formulation excipients are resolved. The assay is linear for tazadolene succinate concentrations equivalent to 50–150% of the assay concentration. Impurities can be quantitated to levels equivalent to about 0.1% by weight with R.S.D. less than 5%. The estimated limit of detection for I is about 2 ng for a 20 μl injection.

INTRODUCTION

Tazadolene succinate (I) is a drug candidate undergoing evaluation for potential use as an analgesic. A method for determining the purity of the bulk drug, levels of impurities in the bulk drug, and potency of clinical formulations was required to support development. A number of investigators have successfully utilized reversed-phase high-performance liquid chromatography (HPLC) for the examination of

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drugs containing aromatic and amine functions[1–6]. Our goal was to develop a simple, rugged assay for release and stability testing, which would provide acceptable linearity, accuracy, precision, and selectivity[7].

EXPERIMENTAL

Chemicals

Acetonitrile and tetrahydrofuran (THF) of UV High-Purity grade were obtained from Burdick & Jackson (Muskegon, MI, U.S.A.). Reagent-grade triethylamine (TEA) (Fisher Scientific, Pittsburgh, PA, U.S.A.) and perchloric acid (Mallinckrodt, St. Louis, MO, U.S.A. were used to prepare the mobile phase. Water was distilled and deionized. Samples of tazadolene succinate, potential process impurities, potential degradation products, and formulation excipients were obtained from Upjohn sources (Table I).

Equipment

A Varian 5060 ternary gradient pump (Walnut Creek, CA, U.S.A.), equipped with a Varian 8055 autosampler and Valco UH6 injector, was used. Detection was accomplished with a LDC UVIII monitor (Riviera Beach, FL, U.S.A.) with a 254-nm filter, a Sargent-Welch XKR recorder (Skokie, IL, U.S.A.) and an in-house VAX-based (Digital Equipment Co., Merrimack, NH, U.S.A.) data acquisition system in our Kalamazoo laboratory. A Spectra-Physics SP 8700 pump (San Jose, CA, U.S.A.), a Waters Assoc. WISP autosampler (Milford, MA, U.S.A.), a Waters Assoc. 441 detector with 254-nm filter, and a Spectra-Physics SP 4200 computing integrator were used in our U.K. laboratories. The column of choice for the assay is a Zorbax C_8, 250 × 4.6 mm I.D. (DuPont, Wilmington, DE, U.S.A.).

Procedure

The mobile phase was prepared by mixing 4.0 ml of TEA with 700 ml of water. The pH of this solution was adjusted to 2.5 by cautious dropwise addition of 70% perchloric acid. Then 250 ml acetonitrile and 50 ml THF were added to this solution and thoroughly mixed (solvent A). Acetonitrile (950 ml) and THF (50 ml) were mixed (solvent B). The solvents were filtered and degassed before use. The acetonitrile–THF levels in solvent A may be adjusted to obtain acceptable separations. Suitable chromatograms are obtained when I is eluted in *ca.* 10 min, testosterone in *ca.* 20 min and I is separated from II (isocratic elution with solvent A). The flow-rate was set at 2.0 ml/min, and *ca.* 20 μl of the preparations was injected.

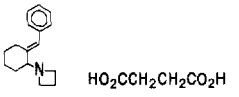
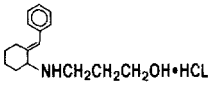
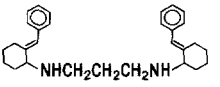
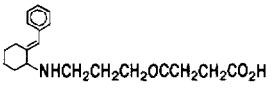
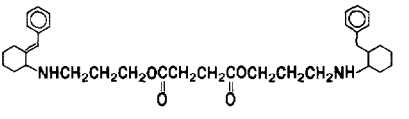
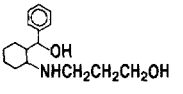
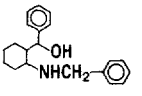
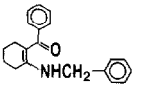
Bulk drug purity was determined with accurately prepared solutions of I at about 0.25 mg/ml concentration in solvent A containing about 0.25 mg/ml testosterone as internal standard. Preparations were analyzed by isocratic elution with solvent A. Purity was calculated by using the internal standard method.

The potency of hard-filled capsules (HFC) was determined by weighing 5 HFC and accurately adding a volume of dichloromethane to produce a concentration of about 2.0 mg/ml, assuming all of I dissolves. These suspensions were shaken vigorously for 20 min and then centrifuged. A 1.0-ml aliquot of supernatant was evaporated to dryness under nitrogen. An accurately measured volume of internal standard solution was added to give a concentration of about 0.25 mg I/ml. The prepa-

TABLE I

CHEMICAL STRUCTURES OF TAZADOLENE SUCCINATE AND RELATED COMPOUNDS

P = potential process impurity, D = potential degradation product, R = structurally related compound.

I		Tazadolene succinate (U-53996H)	
II		Z isomer (U-57700)	(P.D.)
III		U-54142	(P.D.)
IV		U-53034A	(P.D.)
V		U-25583	(P)
VI		U-15811	(P)
VII		2-Benzylidene-cyclohexanone	(P)
VIII		Benzaldehyde	(P)
IX		U-53740 (*trans*)	(R)
X		U-53741 (*cis*)	(R)
XI		U-69609 "dimer"	(P)
XII		U-53034 monoester	(D)
XIII		U-53034 diester	(D)
XIV		U-53033	(R)
XV		U-28208	(P)
XVI		U-62887	(P)

rations were chromatographed isocratically with solvent A. The potency was calculated by using the internal standard method.

Bulk drug impurities were determined with preparations of about 0.5 mg I/ml in solvent A. Blank injections of solvent A were used to determine whether artifacts appeared in the baseline. The preparations were eluted with a gradient program of 0–12 min isocratic 100% A, 12–27 min linear gradient to 25% A–75% B, 27–47 min isocratic 25% A–75% B, 47–52 min linear gradient to 100% A and 52–67 min isocratic 100% A. The chromatogram was monitored for about 55 min. If the retention time of I shifted during a run, the length of the final isocratic step was extended to assure that the column was re-equilibrated between injections. Impurity levels were calculated by using area percent, corrected for response factors.

RESULTS AND DISCUSSION

Tazadolene succinate and related compounds

Tazadolene succinate has the following properties. It is a white to off-white powder, which is soluble in water (55 mg/ml), THF (20 mg/ml), methanol (140 mg/ml), and acetonitrile (4.8 mg/ml). It is a weak base with a pK_b of 4.8, prepared as a racemic mixture. A molar absorptivity of 14 600 l/(mole cm) is observed at 242 nm in ethanol. The partition coefficient for tazadolene base in *n*-octanol–pH 7.0 phosphate buffer is about 12 000. The other compounds listed in Table I have generally similar properties, but conjugation is absent in V, XIV, and XV, the amine group is absent in VI–X and II is a geometric isomer of I. These properties collectively

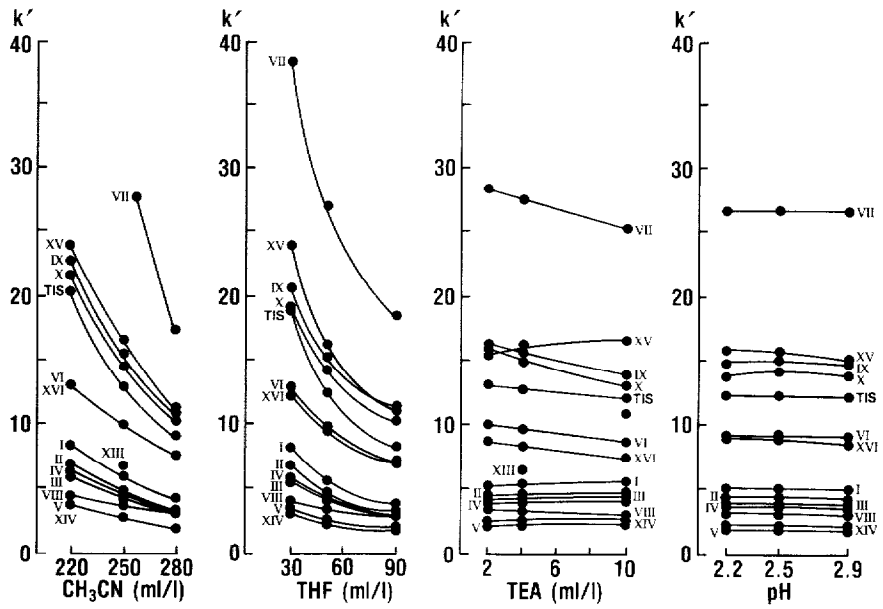


Fig. 1. k′ *vs.* acetonitrile, THF, TEA and pH. In individual isocratic experiments acetonitrile, THF, TEA or pH levels were adjusted as shown. Levels of the other components were acetonitrile (250 ml), THF (50 ml), TEA (2 ml), pH (2.5 with perchloric acid) and water to make 1 l. TIS = testosterone internal standard.

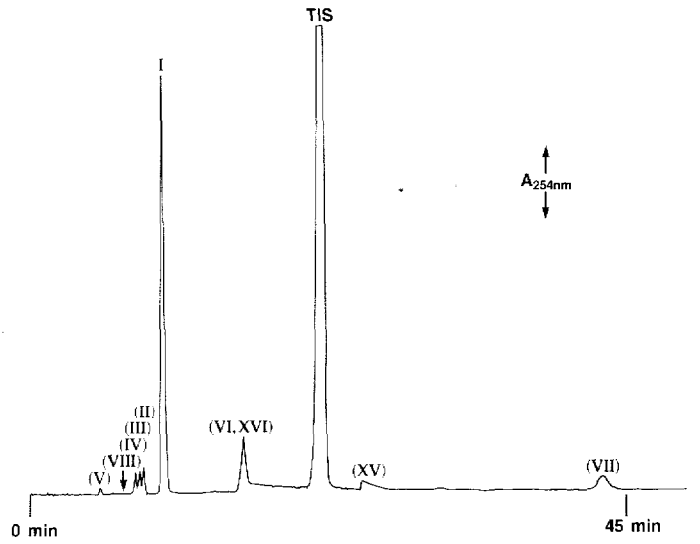


Fig. 2. Isocratic separation of bulk drug, spiked with the compounds indicated. Conditions as described in Experimental. TIS = testosterone internal standard.

suggested that a reversed-phase HPLC system consisting of an alkyl-bonded stationary phase and a binary or ternary mobile phase to provide selectivity, an amine to reduce interactions with residual silanols, and pH adjustment to protonate the amine groups in the samples should be capable of resolving these molecules.

Selectivity and mobile phase effects

Fig. 1 shows the changes in the capacity factors of the samples as a function

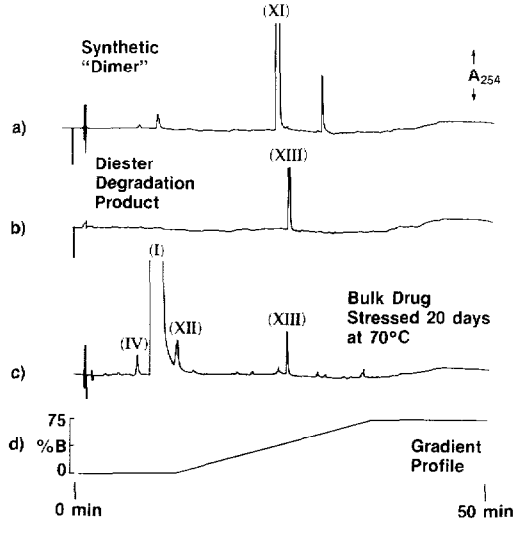


Fig. 3. Gradient chromatograms of (a) synthetic XI, (b) XIII isolated from thermally degraded bulk drug, (c) bulk drug, exposed to 70°C for 20 days and (d) gradient profile, as described in Experimental.

of mobile phase composition. These data indicate that the concentrations of acetonitrile and THF in the mobile phase have the largest impact on determining the retention of any given molecule. This is consistent with the behavior commonly observed for molecules in reversed-phase systems. It was determined that the combination of THF and acetonitrile was necessary to yield resolution of I and II. Separation of these geometric isomers is critical since the *Z* isomer is a potential degradation product. Attempts to resolve them on C_{18} columns with mobile phases containing only acetonitrile or methanol as the "strong" solvent were unsuccessful or difficult to reproduce. It was also observed that changing the acetonitrile–THF ratio could change the order of retention of III and IV (data not in Fig. 1). Mobile phases with a acetonitrile–THF ratio of 4:1 eluted III after I, but did not shift IV significantly. Mobile phases with a 280:20 ratio reversed their positions. However, in both instances the resolution of I from the impurity eluted just after it was inadequate for a routine assay.

The addition of the amine was necessary to bring about elution of I, even with high concentrations of acetonitrile or THF in the mobile phase. Only small concentrations of the amine were needed to insure elution of I (Fig. 1) and they were sufficient to give sharp peaks (Fig. 2). The retention of the samples did not exhibit a strong dependence on TEA concentration above a threshold level of 2.0 ml/l. A concentration was adopted in the mid-range of the concentrations studied to ensure ease of preparation and ruggedness. Atttempts to use larger amine molecules as modifiers, such as N,N-dimethyloctylamine, were unsuccessful, because the column required lengthy equilibration and the column-to-column reproducibility of retention times and elution orders was poor.

Retention was not dependent upon pH over the range studied, as would be expected on the basis of the p*K* values of the compounds studied (Fig. 1). Perchloric acid was chosen for adjusting the pH because it is a strong acid and can form ion pairs that are easily partitioned into hydrophobic phases. Work with other basic compounds in our laboratories suggests that perchloric acid is the acid of choice for

TABLE II

SUMMARY OF QUANTITATIVE DATA FOR PURITY AND POTENCY ASSAYS

	Bulk drug potency	*HFC potency*
Linearity	70–150% of assay concentration*	50–150% of assay concentration*
Average recovery	99.6% for 6 levels in linearity study (R.S.D. = 0.5%)	100.0% for 7 levels in linearity study (R.S.D. = 0.5%)
R.S.D.	0.1–0.5% for 6 replicates	0.1–0.4% for 3 replicates
Limit of detection for I**	2 ng in 20 μl injection	2 ng in 20 μl injection

* See Experimental section.

** Estimated for signal-to-noise ratio of 3 where noise was measured peak to peak.

systems of this type, *i.e.*, the separation of amines on an alkyl stationary phase with a low pH, amine-modified mobile phase.

Inspection of Fig. 1 indicates that the midpoints of each of the curves represents mobile phase compositions that yield good separation of related molecules from I and testosterone within about 40 min with the exception of the large molecules XI and XIII. These conditions were therefore chosen for the bulk drug purity and capsule potency assays.

A linear gradient segment was added to speed the elution of late-eluted compounds. The gradient was started just after I was eluted in order to maintain the resolution described above, especially that of II from I. An example of such a chromatogram is shown in Fig. 3. The late-eluted peaks are considerably sharper as a result of using the gradient (*cf.* Figs. 2 and 3). This provides increased sensitivity for the late-eluted peaks and justifies the use of gradient elution in screening for impurities. We have observed some baseline drift and artifacts in the gradient method and have routinely injected a blank and replicate samples to insure that artifacts are not mistaken for real peaks and *vice versa*. Use of a mixture of acetonitrile and THF in solvent B provided a flatter baseline than that obtained when only acetonitrile was used as solvent B.

Quantitation

A recovery experiment was performed to validate the bulk drug purity and

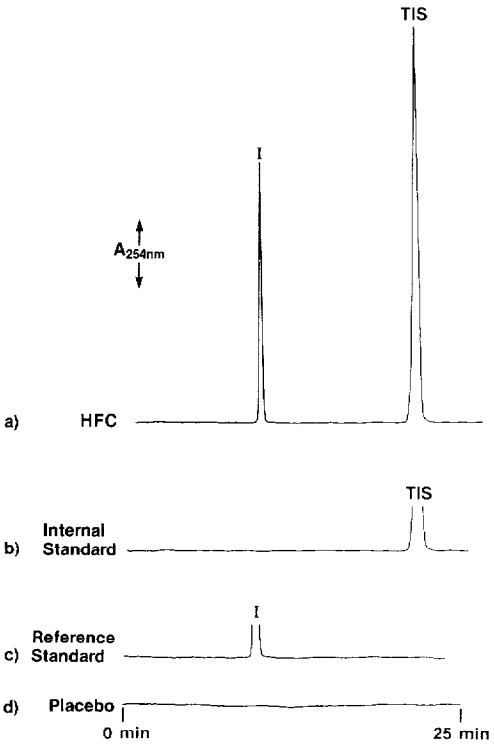


Fig. 4. Isocratic chromatograms of (a) HFC, (b) testosterone, (c) reference standard I and (d) placebo. Conditions as described in Experimental.

capsule potency assays. Table II summarizes the results. These assays give accurate, linear responses without statistically significant bias over the range of concentrations studied. Furthermore, no interferences were observed in the chromatogram of the HFC placebo (Fig. 4). The precision of these potency assays showed less than 1% R.S.D. for replicate assays.

The impurity assay was shown to give linear responses over a range of about 0.02–3% (w/w) when a series of dilutions of synthetic mixtures of the various related compounds and I were injected. Linearity and lack of bias were observed. Table III summarizes the quantitative data for the impurity assay. Limits of detection were estimated at a signal-to-noise level of 3, where noise was measured as baseline peak-to-peak at about 0.00005 a.u.f.s. This was as low as 0.01% (w/w) for the conjugated aromatic compound VIII. Because replicate assays at these levels gave very poor precision (R.S.D. > 10%), the limits of quantitation were estimated for signal-to-noise ratios of 10. This corresponds for most of the impurities to about 0.1% (w/w), which we have chosen as our lower limit for reporting impurities. Peaks detected below this reporting level but above the limit of detection are reported as detected at less than 0.1% (w/w).

Ruggedness/utility

The HPLC system has been used to support preclinical and early clinical studies of tazadolene succinate. It has proved to be reproducible from laboratory to laboratory in terms of resolution, retention, and quantitation. This is due partly to the utilization of DuPont's SAFEGUARD[8] program, which insures that the columns used are packed only with batches of Zorbax C_8, which have been shown to separate I and II. Thermal and UV stress studies have demonstrated that the system

TABLE III

SUMMARY OF BULK DRUG IMPURITIES QUANTITATION

Impurity	*Limit of quantitation* (weight % of impurity)*	*Response factor***	*R.S.D.****
II	0.17	0.53	0.9% (0.68)
III	0.06	1.4	1.0% (0.22)
IV	0.08	0.90	0.2% (0.24)
V	3.0	0.017	3.0% (3.9)
VI	0.11	1.3	3.0% (0.6)
VII	0.63	0.59	1.7% (6.1)
VIII	0.03	3.4	0.7% (0.01)
IX	0.22	1.1	1.1% (0.55)
X	0.27	0.86	2.0% (0.65)
XI	0.08	1.1	3.0% (0.19)
XII	0.08	0.57	3.0% (0.80)
XIII	0.08	1.1	ND
XIV	1.6	0.013	0.4% (5.4)
XV	3.1	0.026	2.7% (10.1)
XVI	0.44	0.36	0.8% (1.1)

* Estimated for signal-to-noise ratio of 10.

** Based on area normalized to I, assigned response factor = 1.0.

*** From triplicate samples with additions at weight% in parentheses, ND = not determined.

resolves the primary degradation products II, IV, XII, and XIII and is, therefore, applicable to stability studies.

CONCLUSIONS

The HPLC system described reliably resolves compounds of interest in relation to tazadolene succinate and provides accurate and precise quantitative information. The *Z*-isomer of tazadolene is consistently resolved. Tazadolene and its related compounds require the presence of a competing amine in low pH mobile phases to insure elution and to give sharp peaks. Perchloric acid is preferred for acidifying the mobile phase. Use of a linear gradient brings about elution of high molecular weight compounds, which are structurally related to tazadolene. The gradient method provides a sensitive method for screening for the presence of impurities in bulk drug.

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