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Determination of pentobarbital and pentobarbital sodium in bulk drug substance and dosage forms by high-performance liquid chromatography

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Abstract

High performance liquid chromatography (HPLC) is used to determine impurities in pentobarbital (I) and pentobarbital sodium (II) and to determine the strength of the drug substance and dosage forms. Separations were achieved using a Nucleosil C-18 column (5 μm) measuring 4.6 mm × 15 cm and an eluent containing 0.01 M phosphate buffer at pH 3.5:acetonitrile (72:28). The column is eluted isocratically and UV detection is used at 214 nm. Impurities are determinable in the drug substance at levels ≥ 0.01%. Assay precision (relative standard deviations) for impurities in I and II ranged from ± 36% to ± 1.3% at levels of 0.01–1.46%. The external standard method is used for quantitating impurities in I and II. The determination of strength in drug substances I and II and in dosage forms (elixir, solution, capsules and suppositories) used the internal standard method. Precision for the strength determination ranged from ± 0.26 to ± 1.6%. The accuracy of the procedure was evaluated by addition and recovery of I and II to placebos. Recoveries were quantitative at 50–150% addition levels. Variation in parameters of the separation were made to evaluate the robustness of the HPLC separations. © 1997 Elsevier Science B.V.

Keywords: Pentobarbital; Barbiturates; High performance liquid chromatography

1. Introduction

Pentobarbital (I) is a well-known barbiturate used as a sedative hypnotic. The official methods for determining I and pentobarbital sodium (II) use titration and gravimetry, respectively [1]. A limit test is included to control the 5-ethyl-5-(1-ethylpropyl) barbituric acid in I and II by melting range of the *p*-nitrobenzyl derivative. Dosage forms of I and II are determined by USP monographs using either a general packed column GC barbiturate assay or by gravimetry. Our goal was to develop stability-indicating procedures for determining impurities in I and II bulk drug substances and to determine strength in the bulk drug substances and marketed dosage forms using a single, reliable chromatographic finish.

A large literature exists for I and II. However, published reports concentrate on toxicological or forensic studies [2–6]. Generally, I is one of many

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Table 1
Robustness data for impurities separation

Buffer pH	Eluent composition		R1[a]	R2[b]	RT (I, min)
	Buffer molarity	%CH_3CN			
3.5	0.01	28	2.1	2.1	15.0
3.5	0.01	26	2.3	—[c]	18.9
3.5	0.01	30	1.8	1.7	12.1
3.7	0.01	28	2.1	2.1	15.0
3.4	0.01	28	2.1	2.0	15.0
3.3	0.01	28	2.1	[c]	15.1
3.5	0.005	28	2.1	2.1	15.0
3.5	0.015	28	2.1	2.1	15.0

[a]R1, R [1] between I and 5-ethyl-5-(1-ethylpropyl) barbituric acid.
[b]R2, R [1] between I and 5-methyl-5-(1,3-dimethylbutyl)barbituric acid.
[c]Not determined.

drugs included in a screen or drug panel. A previous paper described the determination of 3'-hydroxypentobarbital and additional metabolites of I [7]. The 5-ethyl-5-(1-ethylpropyl)barbituric acid content in I has been quantitated by HPLC [8], but the separation is incomplete and the internal standard used could possibly co-elute with other impurities present in the drug substance. The degradation pathways of I, have been described and the separation of the identified melonuric acids were attempted by HPLC [9]. However, the separation is not adequate for quantitating these impurities in I. Intact II has been quantitated in a commercial elixir using a strong anion exchange column and a basic eluent [10]. Paired ion separations of I in dosage forms have been reported using 10 μm irregular C18 packing [11,12]. We focus on using HPLC with modern reverse-phase packings to achieve a unified method for I and II which could control impurities in the drug substance and determine the active drug substance in a wide variety of dosage forms. Optimization of the selectivity for the chromatographic system was of particular importance in this work, since significantly different potential interferences from sample matrices and closely related manufacturing impurities are present.

2. Experimental

2.1. Apparatus

The liquid chromatograph consisted of a model LC-10AD pump and model SIL-10A autosampler (Shimadzu, Koyoto, Japan). A model SPD-10A UV detector and a model C-R7A data handling system (Shimadzu) were used. The chromatographic column was a 4.6 mm × 15 cm Nucleosil C-18 (5 μm diameter, 120 Å pore size) packed by CSC (Chromatography Science Co., St.-Laurent, Quebec, Canada). Nylon membranes (0.45 μm) were used for eluent filtration (Alltech Associates, Deerfield, IL, USA) and PVDF membranes (0.45 μm) were used for sample filtration (Gelman Sciences, Ann Arbor, MI, USA).

2.2. Reagents

The eluent buffer was 0.01 M in monobasic potassium phosphate and was adjusted to pH 3.5 using orthophosphoric acid, both reagent grade (J.T. Baker, Phillipsberg, NJ, USA). The HPLC eluent was a 72:28 mixture of eluent buffer:acetonitrile (HPLC grade, EM Science, Gibbstown, NJ, USA). Tetrahydrofuran (THF) used in some dosage form preparations was HPLC grade (EM Science). 4'-Ethoxyacetophe-

Table 2
Robustness data for strength determination

Buffer pH	Eluent composition		I	
	Buffer molarity	CH_3CN	RT (min)	R
3.5	0.01	28	14.8	14.4
3.5	0.01	29	13.4[a]	14.4
3.5	0.01	30	12.1[a]	14.6
3.5	0.01	27	16.0	14.4
3.5	0.01	26	18.5[a]	14.0
3.6	0.01	28	14.8	14.5
3.4	0.01	28	14.7	14.4
3.5	0.005	28	15.0	14.5
3.5	0.015	28	14.7	14.5

[a]Fails the system suitability requirement.

none used as an internal standard was reagent grade (Aldrich Chemical, Milwaukee, WI, USA). The internal standard solution was 4′-ethoxyacetophenone prepared in acetonitrile at 0.70 mg ml^{-1} concentration. Dosage forms of I and II were manufactured by Abbott Laboratories, North Chicago, IL, USA. The standard used was II which was purchased commercially (Ganes Chemical, Pennsville, NJ, USA). This was screened for impurities; it contained fewer detectable impurities than the current lot of USP reference standard I. The standard contained 0.20% impurities and 3.3% weight loss by TGA (purity factor = 0.965 as II). The standard preparation was made by preparing a stock solution of standard in eluent for an equivalent concentration of I at 1 mg ml^{-1}. For the impurities determination, the stock standard preparation was diluted in the HPLC eluent to contain an equivalent of 5 μg ml^{-1} of I. For the strength determination, the stock standard solution was diluted in the HPLC eluent and combined with an aliquot of internal standard solution to contain an equivalent of 50 μg ml^{-1} of I and 35 mg ml^{-1} of internal standard.

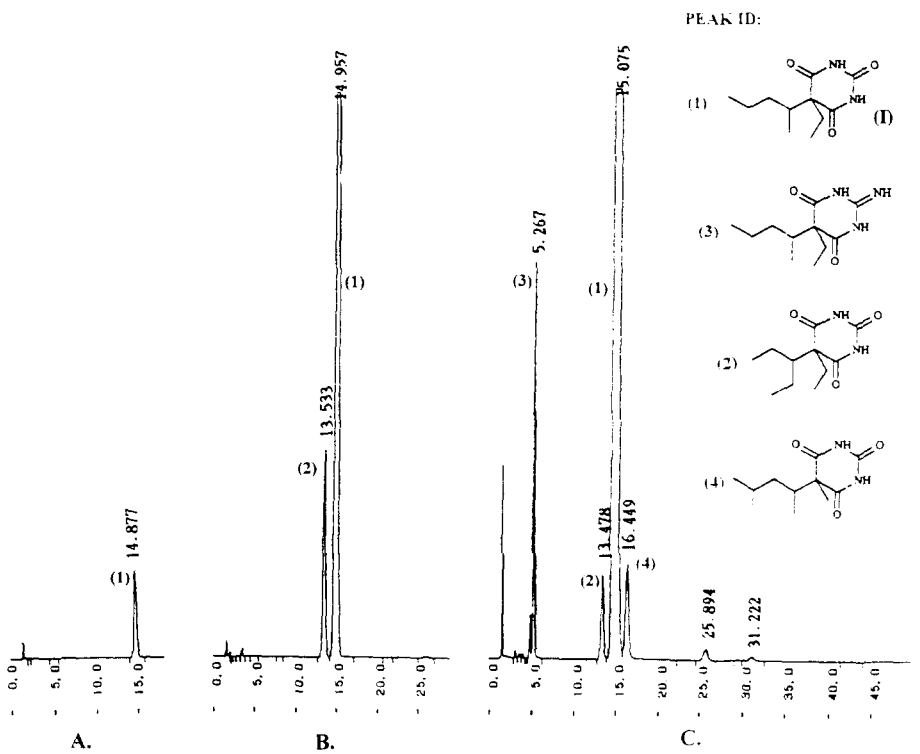

Fig. 1. Typical chromatograms for impurities determination: (A) standard; (B) suitability solution; (C) synthetic mixture of I and possible impurities.

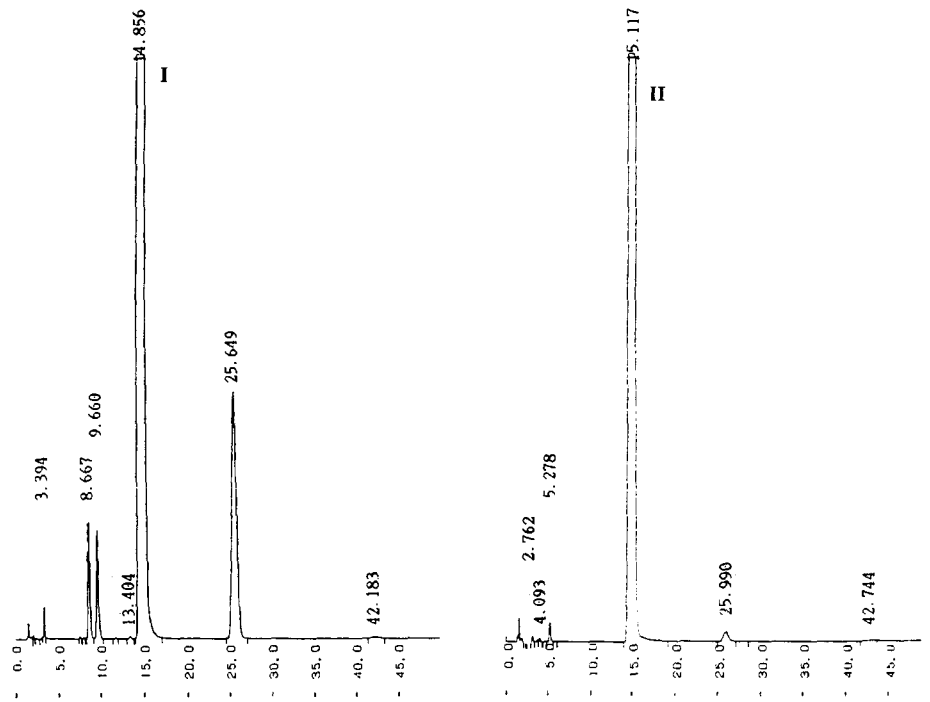

Fig. 2. Impurity profiles of drug substances I and II.

2.3. Sample preparation

For impurities in I and II, solutions of the drug subsance were prepared in the HPLC eluent at 1 mg ml^{-1}. For strength determination in drug substances I and II, the 1 mg ml^{-1} solutions were diluted in the HPLC eluent and combined with an aliquot of internal standard solution to contain an equivalent of 50 μg ml^{-1} as I and 35 μg ml^{-1} of internal standard. For the strength determination in dosage forms, stock solutions were prepared in appropriate solvents. An aliquot was combined with an aliquot of internal standard solution and diluted with HPLC eluent to contain 35 μg ml^{-1} of internal standard and equivalent amounts of I ranging from 30 to 60 μg ml^{-1}. Stock solutions of I elixir (18.2 mg ml^{-1}) and II solution (50 mg ml^{-1}) were prepared in the HPLC eluent. Stock solutions for the II capsules (50 mg and 100 mg) were prepared by extracting the capsule fill with HPLC eluent. The extracts were diluted to known volumes and filtered. Suppositories of II (30, 60, 120, 200 mg) were dissolved in THF/H_2O (9:1) with sonication then diluted to known volumes with THF/H_2O (9:1). The final sample solution was filtered for this preparation to remove small amounts of precipitated wax.

2.4. Typical chromatographic conditions

Column	Nucleosil C-18, 5 μm, 120 Å, 4.6 mm × 15 cm
Eluent	72% (0.01 M phosphate buffer, pH at 3.5) 28% acetonitrile
Detector	214 nm, 0.10 AUFS, Attn = 2 for impurities Attn = 5 for potency
Flow rate	1 ml min^{-1}, approx. 1400 psi backpressure
Temperature	Ambient
Injection volume	50 μl

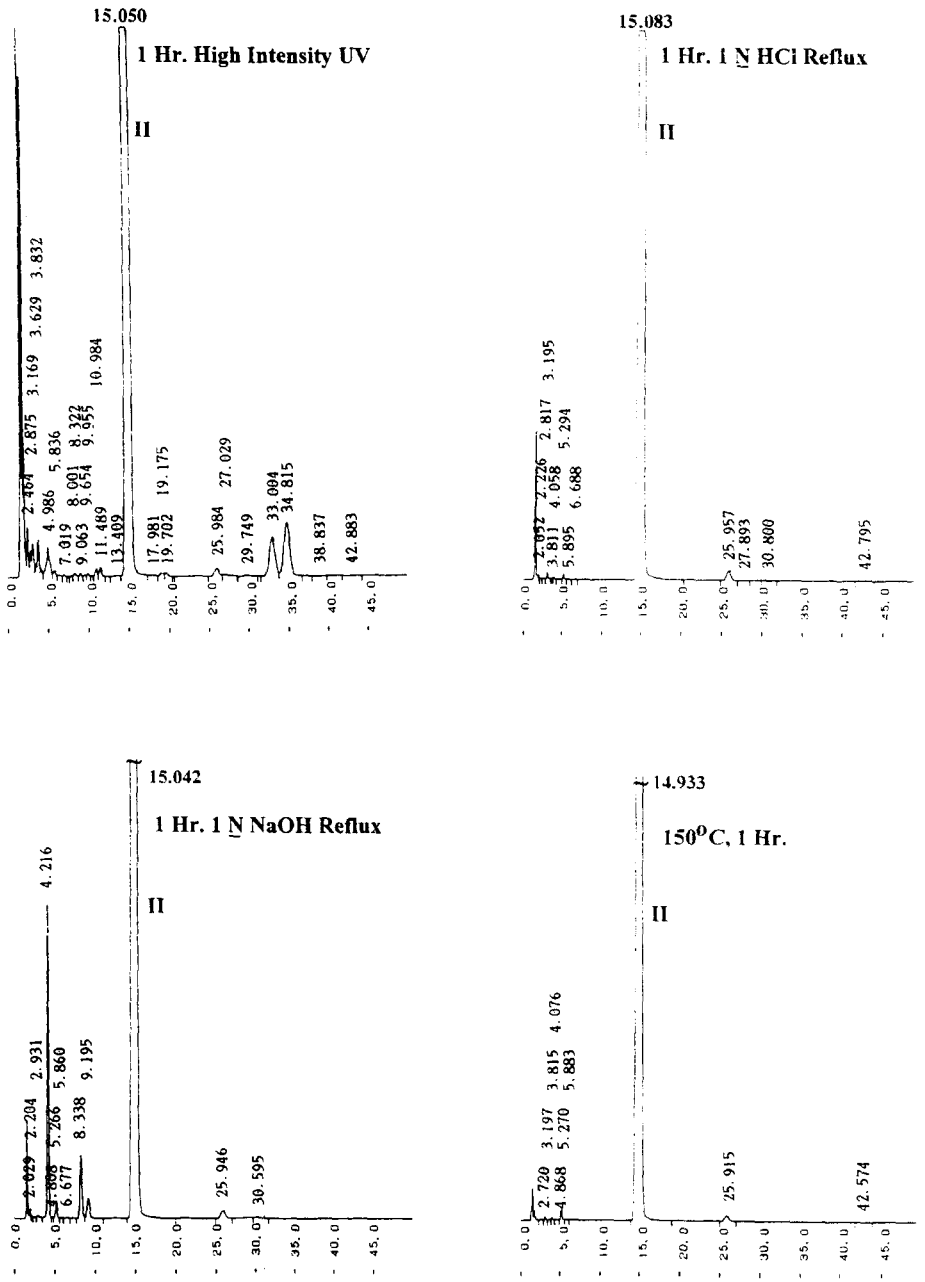

Fig. 3. Chromatograms of II stressed under various conditions.

2.5. Assay procedure

For impurities, replicate injections of the standard preparation were made to obtain three consecutive injections having an R.S.D. of ±2% or lower. Samples were injected and the amount of each impurity was quantitated using peak areas of the individual impurities and the average peak

area of the standard preparation. Periodic reinjection of the standard preparation agreed within 1.5% of the initial mean. For strength determinations a similar routine was used. All quantitation for strength was by the internal standard method using peak area ratios of drug to internal standard.

2.6. System suitability and robustness

For impurities, a system suitability solution was prepared using a lot of I which contained approximately 10% by area of the 5-ethyl-5-(1-ethylpropyl)barbituric acid isomer. The separation of this isomer and I are critical and the quality of this separation generally reflects the resolution of additional closely related impurities and I. Here this critical separation was controlled by eluting I at 14–16 min and maintaining a resolution factor (R, [1]) between 5-ethyl-5-(1-ethylpropyl)barbituric acid and I at ≥ 1.8. These criteria provided acceptable resolution of known impurities eluting closely to I, while maintaining reasonable retention times of more strongly retained impurities.

Table 3
Precision data for impurities in I (USP, Lot H) and II

Impurity	Mean (%)[a]	R.S.D. (±%)	Range (%)
I			
Unknown, RRT = 0.30	0.05	1.2	0.04–0.05
Unknown, RRT = 0.60	0.27	1.9	0.26–0.27
Unknown, RRT = 0.70	0.28	1.7	0.27–0.28
Peak 2, Fig. 1, RRT = 0.90	0.01	—	0.01–0.01
Unknown, RRT = 1.7	1.46	1.3	1.43–1.48
Unknown, RRT = 2.8	0.01	36	0.01–0.02
II			
Unknown, RRT = 0.30	0.02	35	0.01–0.02
Peak 3, Fig. 1, RRT = 0.40	0.07	9.3	0.06–0.08
Unknown, RRT = 1.7	0.09	5.2	0.09–0.10

[a]Mean of ten determinations by multiple analysts, separate days and equipment.

For strength determination, similar criteria were used to assure acceptable chromatographic performance. The chromatographic conditions were controlled to elute I at 14–16 min and the internal standard at 26–31 min. The USP resolution factor (R, [1]) between I and the internal standard was maintained at ≥ 13. Using these conditions, I and the internal standard were separated consistently from the known manufacturing impurities and potential interferences from sample excipients.

Prior to setting the described system suitability requirements, initial variations in the chromatographic conditions were made to assess the robustness of the separation. For impurities, a synthetic solution of I containing approximately 10% by area each of 5-ethyl-5-(1-ethylpropyl)barbituric acid and 5-methyl-5-(1,3-dimethylbutyl) barbituric acid was prepared. These two impurities elute immediately before and after I. The effects of variations in acetonitrile content of the eluent, the buffer concentration and the buffer pH were investigated by chromatographing the synthetic solution. The retention time of I and the resolution between I and the two impurities are tabulated with the eluent variations in Table 1. The data indicate that minor variations in either the buffer pH or ionic strength do not effect the resolution of either the fronting or tailing peaks. The retention and resolution of the system is dependent on the amount of acetonitrile modifier used in the eluent. The data also demonstrate that adequate resolution about I is maintained by controlling the retention time of I as specified in the system suitability.

For strength determinations, the effects of variations in the acetonitrile content of the eluent, buffer concentration and buffer pH were investigated. The retention time of I and the resolution between I and the internal standard are tabulated with the eluent variations (Table 2). As shown, minor variations in the HPLC eluent did not significantly effect the resolution between I and the internal standard. Resolution factors were ≥ 14

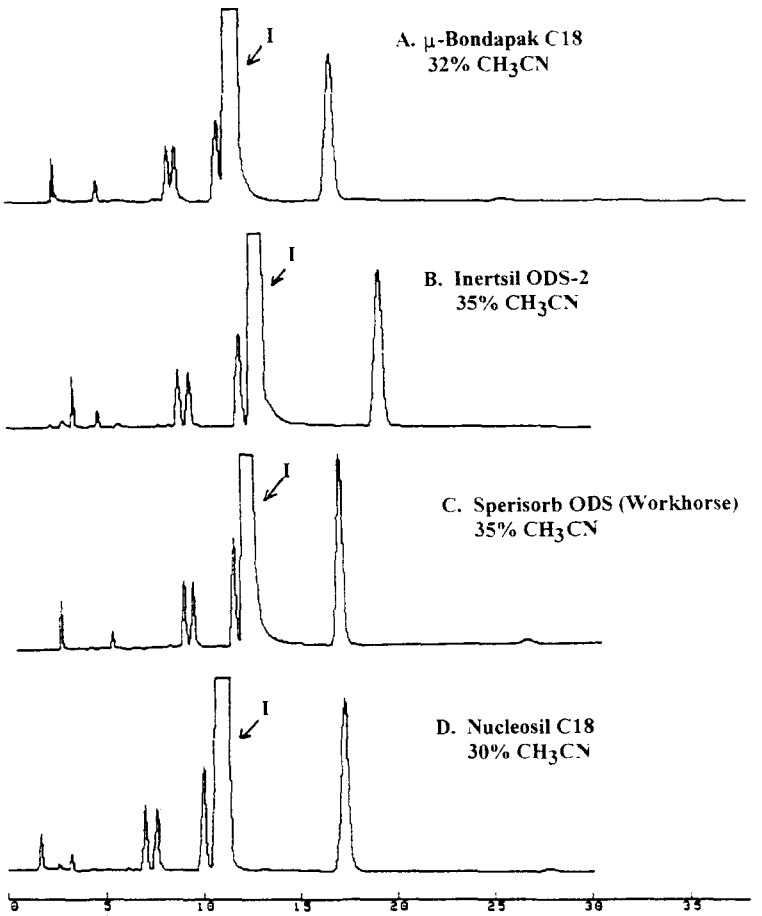

Fig. 4. Chromatograms of a mixture of I and II using different chromatographic columns.

under all conditions. The required retention time for I was met when 27–28% of acetonitrile was contained in the HPLC eluent. Therefore, the amount of acetonitrile modifier must be controlled carefully.

3. Results and discussion

3.1. Impurities

Typical chromatograms used for impurity determinations are shown in Fig. 1. In this figure the standard preparation and resolution mixture are shown. Also presented is a separation for a synthetic mixture containing I and possible manufacturing impurities added at approximately 0.5% (wt/wt). Typical impurity profiles of I and II are shown in Fig. 2. The stability-indicating nature of this separation is demonstrated by stressing the sample preparation of II with high intensity UV light, acid reflux and base reflux. A solid sample was stressed at 150°C for 1 h. As shown in Fig. 3, no significant degradation peaks were observed in the acid reflux or the heat stressed samples. Degradation products were observed, but not identified in the high intensity light and base reflux samples. The sample and standard preparation used in the method are stable for at least 24 h, making the method well-suited for automation.

The detector response is linear from 0.0892 to 89.2 μg ml^{-1} (corresponding to approximately

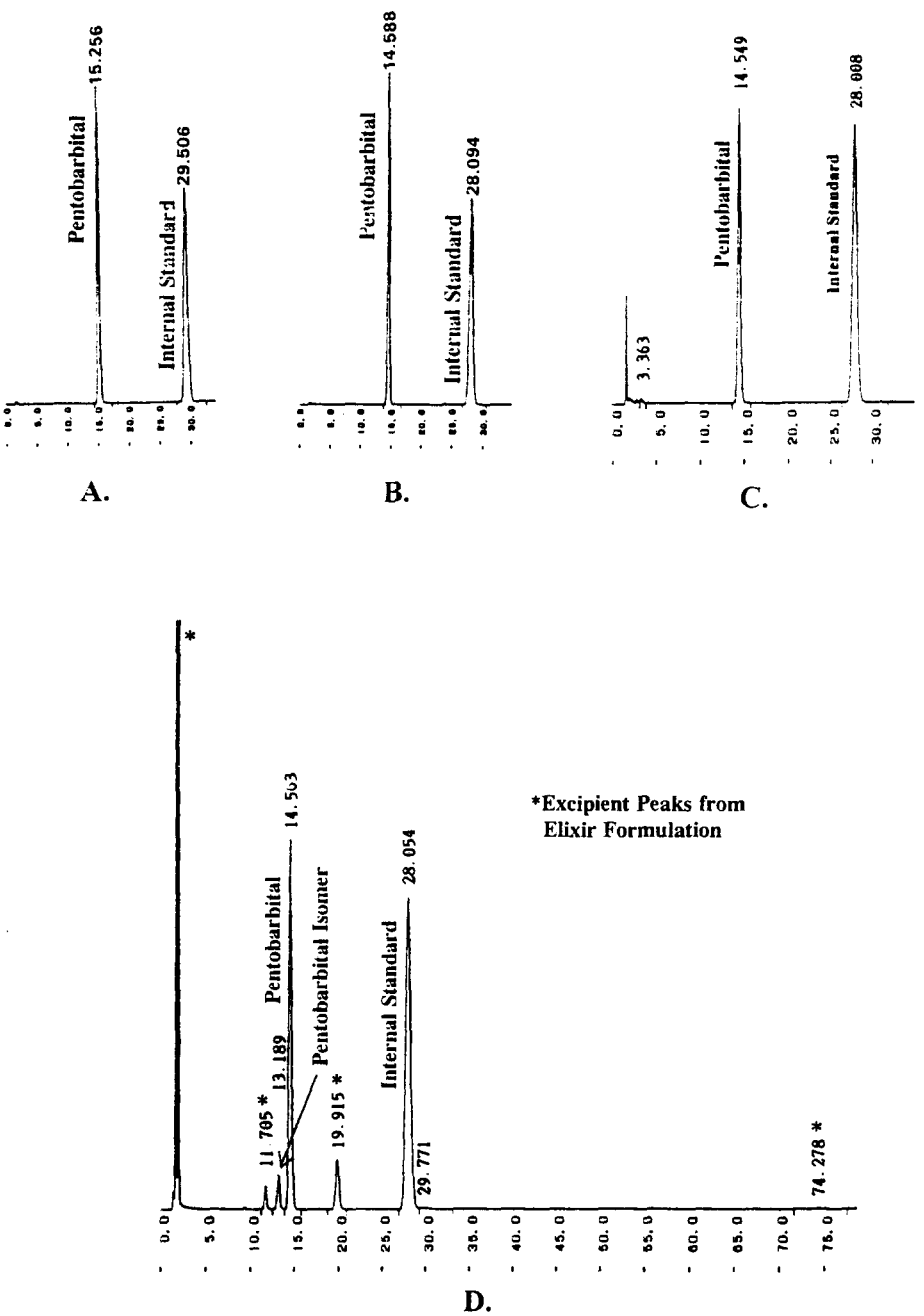

Fig. 5. Typical chromatograms for dosage forms: (A) 50 mg capsule, II; (B) 50 mg ml^{-1} Solution, II; (C) 30 mg Suppository, II; (D) Elixir of I.

0.01–8.9% of the sample preparation) of I. The standard curve for y = peak area response vs. x = concentration, I μg ml^{-1}, has a correlation coefficient greater than 0.9999 and the y-intercept of 14 860 peak area counts is within the 95% confidence interval of the y-intercept.

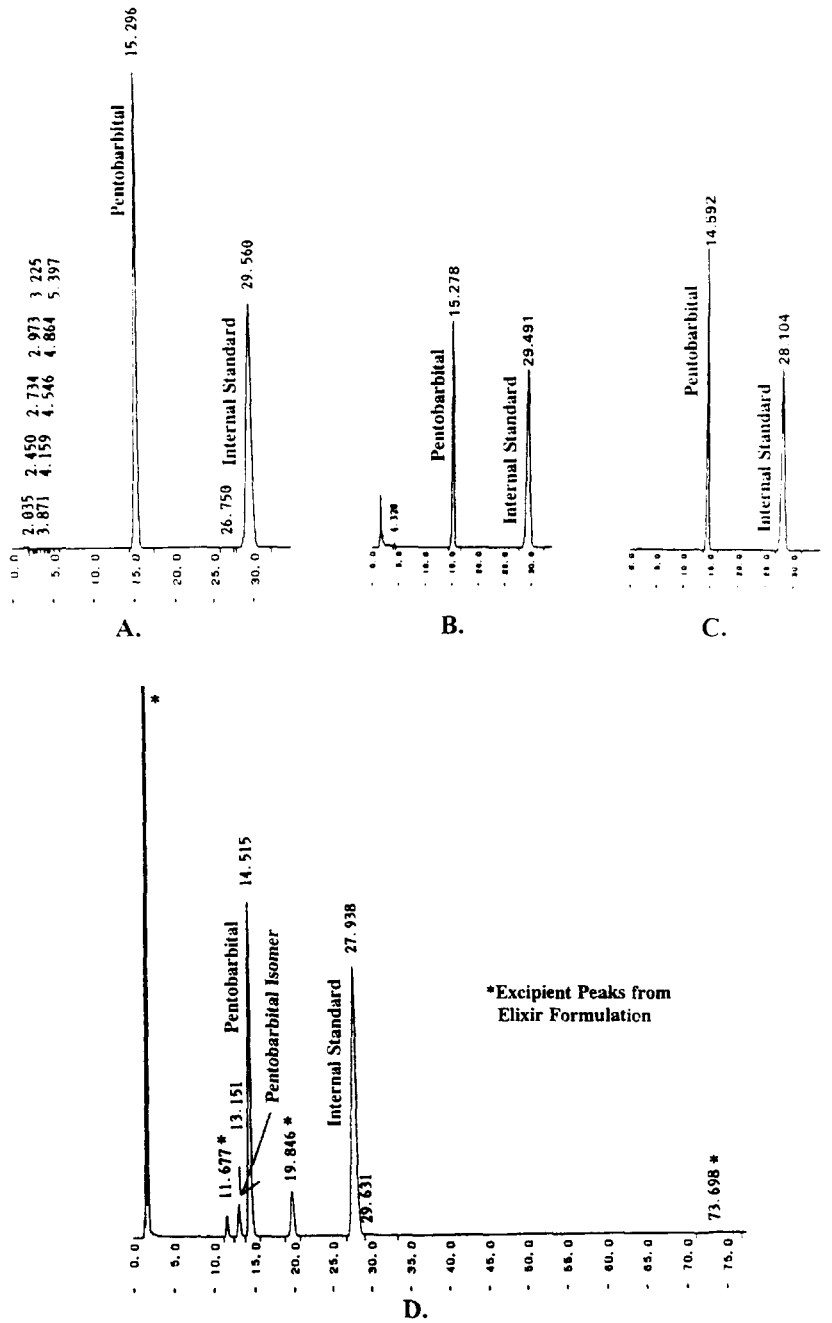

Fig. 6. Typical chromatograms for drug substance and formulation stressed under various conditions: (A) I stressed in high intensity UV light; (B) 50 mg Capsule II, Refluxed in 1 N NaOH; (C) Solution of II Heated at 105°C for 1 h; (D) Elixir of I, heated at 105°C for 1 h.

Table 4
Precision data for I and II in drug substance and dosage forms

Drug	Dosage form	Mean[a]	R.S.D.	Range	N[b]
I	Drug Substance	100.0	±0.30	98.4–100.4	9
II	Drug Substance	98.4	±0.26	97.9–98.6	10
II	Solution (50 mg ml^{-1})	98.2	±0.65	97.2–99.0	10
II	Capsules (50 mg)	97.5	±1.3	96.2–100.6	9
II	Capsules (100 mg)	100.6	±0.47	100.8–102.2	9
II	Suppositories (30 mg)	104.9	±1.3	102.3–106.3	10
II	Suppositories (60 mg)	102.9	±1.6	100.8–105.8	10
I	Elixir (18.2 mg/5 ml)	94.7	±1.3	93.4–96.7	10

[a]Means are expressed as % (anhydrous basis) for drug substance I and II and as % label claim for dosage forms.
[b]Multiple samples by two analysts, separate days and equipment.

Table 5
Addition and recovery data for dosage forms

Drug	Dosage form	% Recovery (addition level)		
		50%	100%	150%
I	Elixir (18.2 mg/5 ml)	100.0, 98.3	100.5, 99.1	99.7, 98.8
II	Suppositories (30 mg)	100.7, 100.0	99.7, 99.7	100.0, 100.4
II	Suppositories (60 mg)	101.8, 102.3	101.5, 100.8	100.3, 100.4
II	Suppositories (120 mg)	103.6, 104.5	101.5, 101.3	98.6, 97.7
II	Suppositories (200 mg)	104.1, 104.3	101.7, 102.5	99.3, 100.0
II	Capsules (50 mg)	100.0	100.4	100.0
II	Capsules (100 mg)	100.6	100.1	100.2
II	Solution (50 mg ml^{-1})	100.0	100.3	99.4

Intermediate precision data for the analytical procedure were generated by three analysts on 3 days using lots of I and II. These materials were selected for this study because they exhibit different impurity profiles with a wide range of impurity concentrations. The results are presented in Table 3. As these data show, precision (R.S.D. values) of the determination varied form ± 36% to ± 1.3% for impurities having means of 0.01 to 1.5%. At the 0.01% level, impurities are readily detectable (typical peaks counts > 1000 with S/N > 10). However, the precision is insufficient for reliable quantitation at this level. At 0.05%, a more acceptable R.S.D. value of ± 12% was obtained, making this a better estimate of the limit of quantitation.

Several additional chromatographic columns were evaluated in this work. A synthetic mixture of I and II were prepared for this evaluation to provide a test mixture containing the maximum number of commonly seen impurities. All columns evaluated were new and were preconditioned with approximately 50 column volumes of acetonitrile/water followed by a similar volume of eluent. The described operating conditions were modified by adjusting the amount of acetonitrile to provide a retention time of I at approximately 11–13 min. Fig. 4 depicts chromatograms and the typical resolution obtained. As demonstrated, the most acceptable combination of resolution and peak shape was achieved by the Nucleosil C18 column which is described in the text.

3.2. Strength determination

For this determination, I and II elute at the

same retention time and have identical peaks shapes. Typical chromatograms for various dosage forms are shown in Fig. 5. Drug substances and dosage forms of I and II were stressed under various conditions (1 N NaOH at reflux, 105°C for 1 h, high intensity UV light) in order to demonstrate the specificity of the assay. Minimal loss of drug was observed under these conditions. These results agree with previous reports [9] in which vigorous conditions were used to degrade I. Fig. 6 demonstrates typical chromatograms for stressed drug substance and formulations. The sample solutions are stable for at least 24 h at room temperature.

Detector linearity was demonstrated by chromatographing standard solutions equivalent to 9.8–97.8 μg ml^{-1} of I (approximately 20–200% of the assay level). A plot of x = concentration (I, μg ml^{-1}) vs. y = peak area ratio gives a regression line which is linear (correlation coefficient > 0.9999) and has a y-intercept within the 95% confidence interval.

Intermediate precision for strength determinations is shown in Table 4. Multiple determinations were made by two analysts on separate days using different equipment and chromatographic columns. As shown, the R.S.D.s for the determination ranged from $\pm 0.26\%$ to $\pm 1.6\%$. The accuracy of the method for dosage forms was demonstrated by addition and recovery experiments of I and II to placebos. Summarized in Table 5 are recovery data from placebos after addition at 50, 100 and 150% of label claim. As shown, recovery ranged from 98.3 to 104.5% at the 50% addition level, 99.1 to 102.5% at the 100% addition level and 98.6 to 100.4% at the 150% addition level.

4. Conclusion

An HPLC method is presented which provides an accurate and precise determination of I and II in drug substances and a variety of dosage forms. A single isocratic system is used to determine impurities in drug substances of I and II. The separations achieved have been optimized for column type and operating conditions. The method is stability indicating, rapid and the chromatographic finish is well-suited for automation.

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References

[1] The United States Pharmacopeia XXIII National Formulary U.S. Pharmacopeial Convention, Rockville, MD.
[2] R.D. Maier and M. Bogusz, J. Anal. Toxicol., 19 (1995) 79–83.
[3] M. Bogusz and M. Erkens, J. Chromatogr., 674 (1994) 97–126.
[4] O.H. Drummer, A. Kotsos, I.M. McIntyre, J. Anal. Toxicol., 17 (1993) 225–229.
[5] H.H. Maurer, J. Chromatogr., 530 (1990) 307–326.
[6] K. Jinno, M. Hayashida and T. Watanabe, J. Chromatogr. Sci., 28 (1990) 367–373.
[7] P.L. Cary and B.E. Pape, J. Chromatogr., 275 (1983) 107–114.
[8] J. Hoogmartens, E. Roets and H. Vanderhaeghe, J. Chromatogr., 219 (1981) 431–435.
[9] V.D. Reif, K.L. Kaufmann, N.J. DeAngelis and M.C. Frankhouser, J. Phar. Sci., 75 (1986) 714–716.
[10] R.W. Roos, J. Pharm. Sci., 61 (1972) 1979–1984.
[11] J.H. Block, H.L. Levine and J.W. Ayres, J. Pharm. Sci., 68 (1979) 605–608.
[12] F.B. Ibrahim, J. Liq. Chromatogr., 16 (1993) 2835–2851.