Journal of Chromatography A, 658 (1994) 489–496
Elsevier Science B.V., Amsterdam

CHROM. 25 456

Preparative liquid chromatographic isolation of unknown impurities in Arbidol and SI-5

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ABSTRACT

The identification of low level impurities is a necessary step in the drug development process. This paper describes the methodology used for the isolation of low level impurities in Arbidol and its immediate precursor, SI-5. Preparative liquid chromatography was used to successfully isolate twelve low-level impurities from Arbidol and SI-5. The isolated impurities were of sufficient quantity and purity for subsequent identification.

INTRODUCTION

The identification of all synthetic impurities greater than 0.1% in a drug substance is part of the drug development process. Isolating and identifying low level impurities serves two purposes. First, identification can help determine the origin of the impurities, allowing the synthesis to be modified to reduce the percentage of the impurity in the final product. Second, isolation and identification allows the level in the final product to be accurately determined. There are two different techniques which can be used for identification of low level impurities. The first involves an analytical separation followed by a spectroscopic identification. Examples of this approach include GC–MS [1] and LC–MS [2]. The second approach is the use of preparative liquid chromatography to isolate the component of interest [3–5]. The isolated material is then identified using spectroscopic techniques. The use of preparative chromatography to isolate low-level impurities in bulk chemical is the preferred choice at Searle.

In 1991, Searle contracted with the Russian Federation to synthesize multi-ton quantities of Arbidol (Fig. 1), a drug used for the treatment of influenza. Searle modified the synthesis developed by Russian chemists to improve yields and to eliminate potentially hazardous reagents. The Arbidol prepared using the modified synthesis contained new impurities. Isolation and identification of these unknown impurities was needed so their origin could be determined.

Two different approaches can be used for the isolation of low level impurities. The first involves the scaleup of an analytical HPLC method [6–8]. Throughout this paper this technique is referred to as preparative HPLC. The second is to develop a TLC separation, which is scaled up to a preparative chromatographic method [9–11]. In this paper this technique is referred to as column chromatography.

This paper reports on the isolation of all impurities greater than 0.1% in Arbidol and in SI-5, the immediate precursor to Arbidol. For the isolation, an approach that combined both column chromatography and preparative HPLC

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0021-9673/94/$07.00
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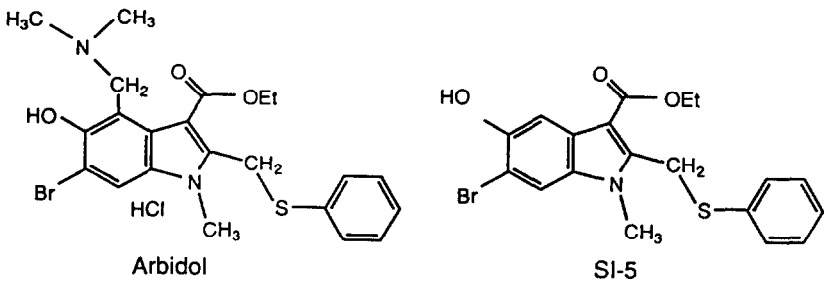


Fig. 1. Structures of Arbidol and SI-5.

was followed. Initial purifications using TLC scaleup were performed to enrich the impurities. During this step and in subsequent steps, some of the impurities were isolated in sufficient quantity and purity for identification. In addition some of the impurities were isolated using preparative HPLC.

EXPERIMENTAL

Equipment

The analytical chromatograph consisted of a Spectra-Physics (San Jose, CA, USA) SP8700 pump or a Waters Associates Model 590 solvent-delivery system and a U6K injector or Waters Intelligent Sample Processor (Milford, MA, USA), a Kratos Model 783 variable-wavelength detector (Ramsey, NJ, USA), a Linear Model 585 recorder (Hackensack, NJ, USA), and Digital Equipment Corporation VAX 11/785 computer with Searle chromatography data system.

The large-scale preparative liquid chromatograph was either a SepTech Model ST/800A or ST/800C (Wakefield, RI, USA). The small-scale preparative liquid chromatograph consisted of two Beckman Model 101 pumps with preparative heads, a Model 165 variable-wavelength detector with a 5-mm semi-preparative flowcell, a Model 450 data system/controller (Berkeley, CA, USA) and a Kipp and Zonen Model BD41 two channel recorder (Delft, Netherlands). A Rheodyne Model 7125 syringe loading sample injector (Cotati, CA, USA) equipped with a 10-ml loop (Valco, Houston, TX, USA). The column effluent was fractionated using a Gilson Model FC220 fraction collector (Middleton, WI, USA). The preparative columns varied in size from 60 mm × 10 mm I.D. to 500 mm × 2 in. I.D. (1 in. = 2.54 cm) and were obtained from a variety of sources.

Materials

The analytical HPLC columns were obtained from either Millipore, Waters Corporation (Milford, MA, USA) or DuPont Medical Products–Biotechnology Systems (Wilmington, DE, USA). The bulk packings were Merck silica gel 60, 40–63 μm, 60 Å irregular silica gel from EM Science (Cherry Hill, NJ, USA). All chemicals for purification were synthesized in the laboratories of Searle (Skokie, IL, USA). The solvents and reagents were reagent grade or better and obtained from a variety of sources.

TLC separations were achieved on Merck silica gel 60 F_{254} TLC plates (Darmstadt, Germany). Spots were detected by irradiating the plate using UV light at 254 nm or putting the plate in an iodine chamber, followed by irradiation using UV light at 254 nm.

The aqueous portion of the mobile phase used for analytical HPLC analysis of Arbidol was prepared by accurately weighing 3.45 g of ammonium dihydrogenphosphate and 1.45 g of 1-pentanesulfonic acid sodium salt into 900 ml of HPLC-grade water. pH was adjusted to 2.5 with phosphoric acid. Volume was brought to 1 l with additional water. The buffer solution was mixed well and filtered through a 0.45-μm nylon filter.

RESULTS AND DISCUSSION

Isolation of Arbidol impurities

HPLC analysis of the initial Arbidol lots synthesized at Searle using the new synthesis showed the presence of five low-level impurities

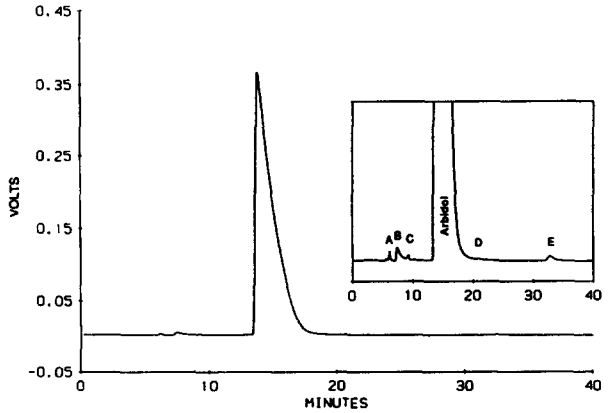


Fig. 2. Analytical HPLC separation of Arbidol. Analysis conducted on Nova-Pak Phenyl, 15 cm × 4.6 mm I.D., detection at 254 nm, 0.1 AUFS. Mobile phase, methanol–pentane sulfonate/phosphate buffer pH 2.5 (75:25), flow-rate 0.6 ml/min.

which were not seen in the lots synthesized in the Russian Federation. These impurities were designated unknowns A, B, C, D and E (Fig. 2). The area percentages of these impurities ranged from 0.03 to 0.5% in the lots synthesized at Searle. For our work, a sample of Arbidol mother liquors from the recrystallization was available. HPLC analysis of this sample showed a comparable impurity profile (Fig. 3), with a few additional impurities. Since the impurities of interest were present at larger percentages (0.3 to 0.9%) the time required for isolation would be reduced compared to the Arbidol lots.

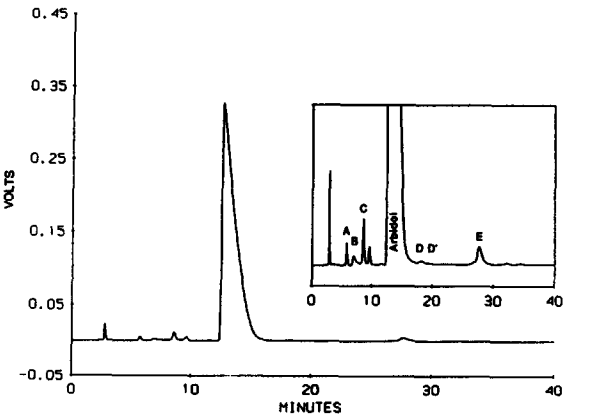


Fig. 3. Analytical HPLC separation of Arbidol mother liquor sample. Analysis conducted on Nova-Pak Phenyl, 15 cm × 4.6 mm I.D., detection at 254 nm, 0.1 AUFS. Mobile phase, methanol–pentane sulfonate/phosphate buffer pH 2.5 (75:25), flow-rate 0.6 ml/min.

Prior to the isolation work, extensive normal-phase TLC method development was performed on the Arbidol mother liquor sample. The system which gave the best separation of Arbidol and the other components was isopropanol–methylene chloride–ammonium hydroxide (3:97:0.15, v/v/v) on Merck TLC plates. Arbidol had an R_F of *ca.* 0.3 with both polar and non-polar impurities detected by TLC. The objective for this purification was the removal of Arbidol and enrichment of the impurities. A 21-g amount of mother liquors was chromatographed using an isopropanol–methylene chloride–ammonium hydroxide step gradient. Due to poor solubility of the hydrochloride salt in the preparative mobile phase, the sample was applied to the column using the solid injection technique [12]. The separation was monitored by TLC. Final fractions were analyzed using the HPLC conditions shown in Fig. 2. Correlations between the spots detected in the TLC method and the peaks present in the HPLC analysis were made. The impurities of interest were enriched during the first purification. The next step in the isolation process was the further purification of the enriched unknowns to generate material suitable for identification. The following paragraphs will discuss the purifications performed to isolate the individual impurities.

The initial chromatography produced a fraction enriched in unknown A. The main component in this enriched fraction, beside unknown A, was Arbidol. Additional TLC method development showed that replacing isopropanol with methanol improved the separation between Arbidol and unknown A. The developed method was methanol–methylene chloride–ammonium hydroxide (3:97:0.2, v/v/v) on Merck TLC plates. This method was scaled up to preparative loadings. Due to the small separation seen between unknown A and Arbidol, a low loading of 10 mg sample per gram packing was used. A 3.8 mg amount of unknown A, with a HPLC purity of 79% (area percent) was generated. The structure of unknown A as determined by NMR and MS is shown in Fig. 4.

Although unknown A and B are closely elut-

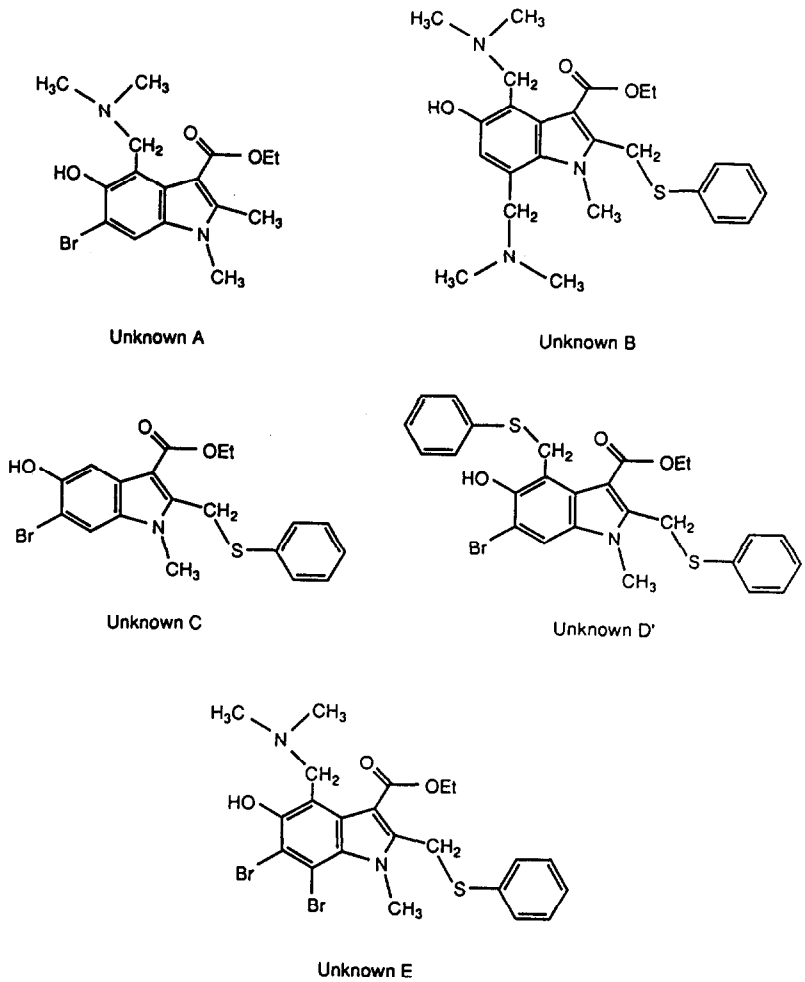


Fig. 4. Structures of Arbidol impurities, unknown A, B, C, D' and E.

ing by HPLC, unknown B required a more polar mobile phase (14% *vs.* 3% methanol) than unknown A when isolated using normal-phase chromatography. Unknown B was isolated from an enriched fraction using a methanol–methylene chloride–ammonium hydroxide step gradient on Merck silica gel. This purification generated 32 mg of unknown B with an HPLC purity of 93% (area percent). The structure of unknown B as determined by NMR and MS is shown in Fig. 4.

The isolation of unknown C was more practical from a different mother liquor sample than that used for the isolation of the other impurities. This mother liquor sample contained 28% (area percent) unknown C. While this mother liquor sample was not useful for the isolation of the other unknowns, its composition made it extremely useful for the isolation of unknown C. The sample was purified using an isocratic methylene chloride mobile phase. This purification generated 108 mg of unknown C with an HPLC purity of 97% (area percent). The structure of unknown C, as determined by NMR and MS is shown in Fig. 4.

The initial purification of the mother liquor sample only slightly enriched unknown D. Combined with the fact that unknown D is one of the lowest impurities (by percentage) present in Arbidol, its isolation was not pursued.

Initial chromatography of the Arbidol mother liquor sample resulted in a fraction enriched in

unknown D′ (Fig. 3). Although this was not one of the desired impurities, since it was enriched to such a high percentage (40%), a decision was made to isolate the impurity. A step gradient of methylene chloride–hexane to isopropanol–methylene chloride generated 7 mg of unknown D′ with an HPLC purity of 94% (area percent). The structure of unknown D′ as determined by NMR and MS is shown in Fig. 4.

The final unknown isolated from the Arbidol mother liquor sample was unknown E. The enriched fraction contained *ca.* 2% unknown E. The remainder of the sample was Arbidol. By TLC, little separation was seen between unknown E and Arbidol. TLC method development was performed to improve the separation. The best method developed was ethyl acetate–ammonium hydroxide (99.5:0.5, v/v) on Merck TLC plates. While the new TLC method had slightly improved separation, several purifications were still required to isolate unknown E in sufficient purity. From these purifications, 41 mg of unknown E with an HPLC purity of 85% was generated. The structure of unknown E as determined by NMR and MS is shown in Fig. 4.

Isolation of SI-5 impurities

HPLC analysis of the initial lots of SI-5 synthesized in Skokie using the new synthesis showed the presence of four major impurities. These impurities were designated unknowns A, B, C and D (Fig. 5). The area percentages of these impurities ranged from 1.0 to 2.7%. Since SI-5 was the first process intermediate which showed appreciable quantities of any impurities, their isolation and identification was required to determine their origin.

As with Arbidol, a mother liquor sample which contained increased quantities (from 3.9 to 30.4%) of the impurities was used for the isolation work. The mother liquor sample contained a comparable impurity profile with a few additional impurities (Fig. 6). The 37-g sample of SI-5 mother liquors was fractionated using a step gradient of isopropanol–methylene chloride generating fractions enriched in the desired unknowns. Three additional impurities were isolated from the mother liquor sample (unknowns 1, 2 and 3, see Fig. 6). The amount

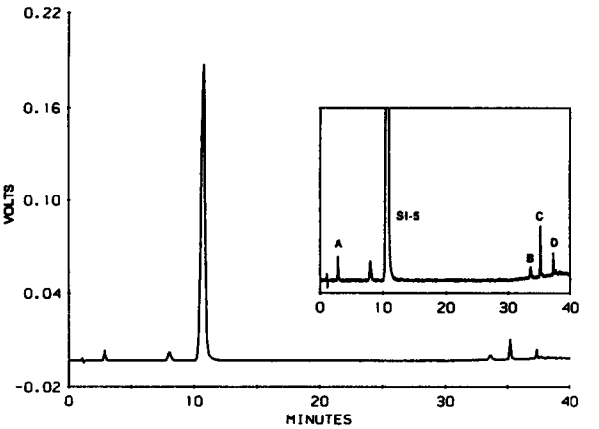


Fig. 5. Analytical HPLC separation of SI-5. Analysis conducted on Nova-Pak C_{18}, 15 cm × 3.9 mm I.D., detection at 310 nm, 0.1 AUFS. Mobile phase, methanol–water (65:35) (25 min), linear gradient to 100% methanol in 10 min, hold at 100% methanol for 5 min, flow-rate 1.0 ml/min.

isolated and HPLC purity (area percent) of the unknowns are: unknown 1, 1.3 g, 98%; unknown 2, 500 mg, 95%; and unknown 3, 430 mg, 85%. The structure of unknowns 1, 2 and 3 as determined by NMR and MS is shown in Fig. 7.

An enriched sample containing unknown C and SI-5 was generated during the initial chromatography of the SI-5 mother liquor sample. A TLC method using a tetrahydrofuran–hexane (50:50) solvent system on Merck TLC plates was

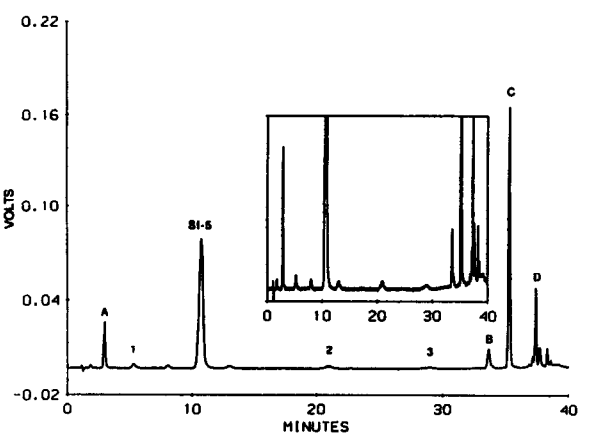
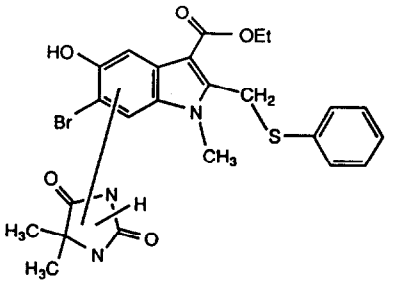
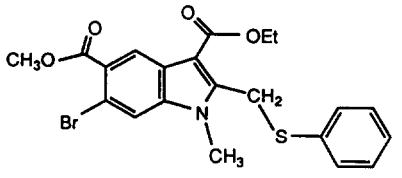


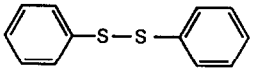
Fig. 6. Analytical HPLC separation of SI-5 mother liquor sample. Analysis conducted on Nova-Pak C_{18}, 15 cm × 3.9 mm I.D., detection at 310 nm, 0.1 AUFS. Mobile phase, methanol–water (65:35), (25 min), linear gradient to 100% methanol in 10 min, hold at 100% methanol for 5 min, flow-rate 1.0 ml.



Unknown 1



Uknown 2



Unknown 3

Fig. 7. Structures of SI-5 impurities, unknowns 1, 2 and 3.

developed. Several attempts were made to separate the two compounds by scaling up the TLC system. Severe tailing of both compounds prevented the isolation of unknown C. The tailing was traced to the low solubility of the sample in the mobile phase. An analytical HPLC method using a DuPont Pro 10 C_8 column and a methanol–water mobile phase was scaled up to preparative loadings. The poor separation obtained was traced to the low solubility of the compounds in the methanol–water mobile phase. Additional method development was performed using a tetrahydrofuran based mobile phase since the compounds had increased solubility in tetrahydrofuran. An analytical HPLC separation was developed on a Zorbax Pro 10 C_8 column with a tetrahydrofuran–water (50:50, v/v) mobile phase (Fig. 8). This method was directly scaled up to a preparative loading of 100 mg on a 25 cm × 21.5

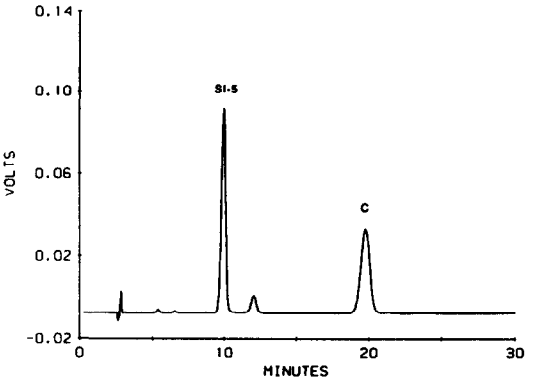
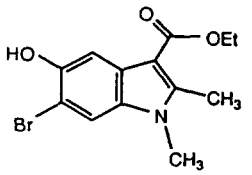


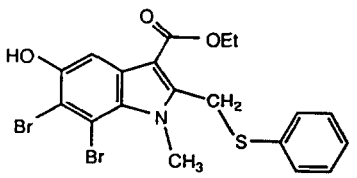
Fig. 8. Analytical HPLC separation of SI-5 fraction enriched in unknown C. Analysis conducted on Zorbax Pro 10 C_8, 25 cm × 4.6 mm I.D., detection at 310 nm, 0.1 AUFS. Mobile phase, tetrahydrofuran–water (50:50), flow-rate 1.0 ml/min.

mm I.D. column. From this purification, 64 mg of unknown C with an HPLC purity of 96% (area percent) was isolated. The structure of unknown C as determined by NMR and MS is shown in Fig. 9.

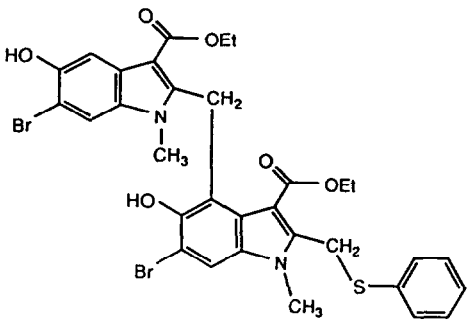
Based on the knowledge gained during the isolation of unknown C, a reversed-phase method employing tetrahydrofuran was used to isolate unknowns A, B and D. The analytical HPLC separation of the fraction enriched in unknowns A, B and D is shown in Fig. 10. This method was directly scaled up to a preparative loading of 100 mg on a 250 mm × 21.5 mm I.D. column. This purification resulted in 54 mg of unknown A with an HPLC purity of 94% (area percent) and 8 mg of unknown B with an HPLC purity of 80% (area percent). The structure of unknowns A and B as determined by NMR and MS is shown in Fig. 9. Unfortunately it was discovered that unknown D had degraded during workup. The degraded material still contained some unknown D and was repurified using conditions identical to the first purification. In an attempt to limit degradation, the wet fractions were quickly worked up in the absence of heat, and the dried material was stored at −76°C. This purification resulted in the isolation of 8 mg of unknown D with an HPLC purity of 80% (area percent) and 14 mg of the degradation product with an HPLC purity of 93% (area percent). The



Unknown A



Unknown B



Unknown C

Fig. 9. Structures of SI-5 impurities, unknowns A, B and C.

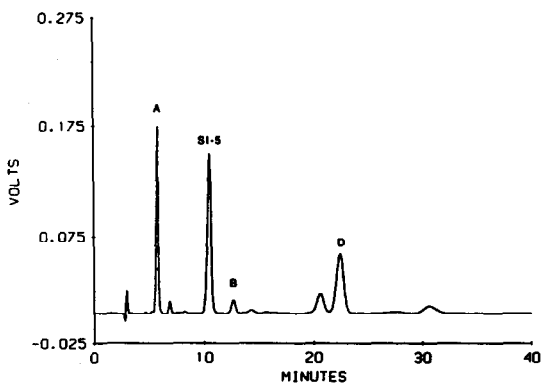
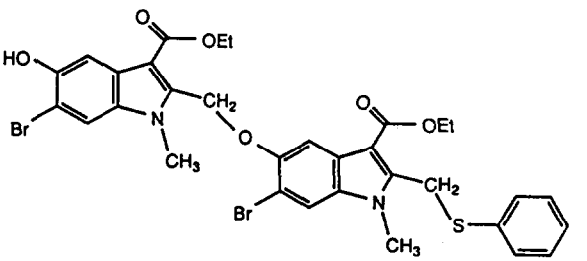
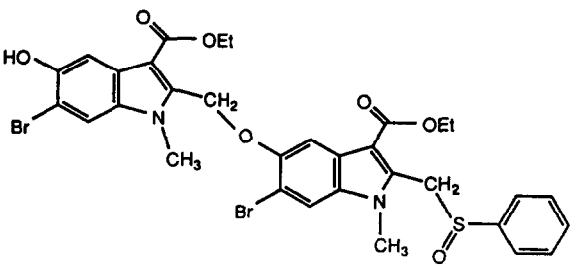


Fig. 10. Analytical HPLC separation of SI-5 fraction enriched in unknown A, B and C. Analysis conducted on Zorbax Pro 10 C_8, 25 cm × 4.6 mm I.D., detection at 310 nm, 0.1 AUFS. Mobile phase, tetrahydrofuran–water (50:50), flow-rate 1.0 ml/min.



Unknown D



Unknown D Degradation Product

Fig. 11. Structures of SI-5 impurity unknown D and degradation product of unknown D.

structure of unknown D and its degradation product as determined by NMR and MS is shown in Fig. 11.

CONCLUSIONS

Preparative chromatography is a powerful technique for the isolation of low-level impurities in drug substances. A multifaceted approach, employing both column chromatography and preparative HPLC was used for the isolation of twelve impurities in Arbidol and SI-5. The isolated impurities were of sufficient quantity and purity for subsequent structure elucidation.

ACKNOWLEDGEMENTS

The authors thank H. Bush, C. Kim, P. Finnegan, J. Hribar, K. Paul and J. Wysocki for their technical support.

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