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Effects of the pre-column in automated on-column injection capillary gas chromatography

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ABSTRACT

In this work, we investigated the effects of pre-columns and press-fit connectors on automated cold on-column injection capillary gas chromatography. Verapamil, a calcium channel blocking vasodilator used in the treatment of angina, arrhythmias and hypertension, and norverapamil, an active metabolite, were used as model compounds in these investigations. Wide-bore fused-silica tubing deactivated with OV-1701-vinyl was also studied with respect to its suitability as pre-column material. The detector response of verapamil *versus* an internal standard was consistent at μg/ml and ng/ml levels, while that of norverapamil decreased with the amount injected. However, the decrease in response of norverapamil appeared to be unrelated to the presence of a pre-column or press-fit connector in the chromatographic system.

INTRODUCTION

Cold on-column injection using traditional narrow-bore (0.2–0.32 mm I.D.) fused-silica (FS) capillary columns with modern automatic injection systems requires the attachment of a pre-column consisting of short section of wide-bore (0.52 mm I.D.) FS tubing. In a recent article Grob and Schilling [1] reviewed the current literature on these uncoated pre-columns or retention gaps and listed three additional purposes which included: (1) analysis of dirty samples; (2) reconcentration of bands broadened in space; and (3) high-oven-temperature on-column injection. In 1986, Rohwer *et al.* [2] introduced the press-fit connector. The introduction of the press-fit type connectors has revolutionized the attachment of pre-columns to coated capillary columns [1,2]. This is especially true when the I.D. of the pre-column differs from the I.D. of the coated capillary column. Recent reports suggest that the press-fitt connector may soon replace the previously used butt connectors and fused connectors [1,3].

Verapamil (Fig. 1), a calcium channel blocking vasodilator used in the treatment of angina, arrhythmias and hypertension, undergoes significant first-pass hepatic metabolism [4]. The N-demethylated metabolite, norverapamil (Fig. 1), retains pharmacological activity and may reach levels in serum equal to or greater than the parent compound. Determination of verapamil in human serum has been accom-

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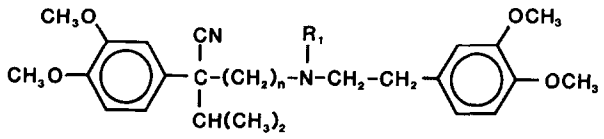

R_1 = CH_3, n = 3 Verapamil
R_1 = H, n = 3 Norverapamil
R_1 = CH_3, n = 2 D-517 (internal standard)

Fig. 1. Structures of verapamil, norverapamil and D-517.

plished using a variety of techniques including gas chromatography (GC) with nitrogen–phosphorus detection (NPD) [5–8]. The determination of norverapamil by GC using NPD has been relatively unsuccessful. However, the reasons for this failure have not been thoroughly investigated.

Capillary GC using cold-on column injection is the preferred GC methodology in terms of reproducibility and linearity of response [9]. In addition, cold on-column injection places the least stress on sensitive solutes of any injection mode. Sample components are restricted exclusively to the environment of the column itself until they reach the detection system [9]. However, as discussed above, automation of on-column injection requires the addition of a pre-column when narrow-bore (0.2–0.32 mm I.D.) columns are used. In this work, we investigated the effects of the pre-column and the press-fit connectors on the analysis of verapamil and norverapamil using D-517 {Fig. 1, α-isopropyl-α-[(N-methyl-N-homoveratryl)-β-aminoethyl]-3,4-dimethoxyphenylacetonitrile} as an internal standard (IS). The difference in the reactivity of verapamil and its active metabolite, norverapamil, toward the GC system was also studied. The FS pre-column (0.52 mm I.D.) used in this work was deactivated with OV-1701-vinyl (7% phenyl, 7% cyanopropyl, 86% methyl silicone with 1% of these groups replaced by vinyl groups) using the method of Arrendale and Martin [10]. To our knowledge, deactivated pre-columns of this type have not been investigated previously.

EXPERIMENTAL

Materials

Verapamil, norverapamil and D-517 (hydrochlorides, Fig. 1) for use as standards were kindly provided by Knoll (Whippany, NJ, U.S.A.). Fused-silica (FS) capillary tubing (0.32–0.52 mm I.D.) was obtained from Polymicro (Phoenix, AZ, U.S.A.); OV-1701-vinyl from Alltech (Arlington Heights, IL, U.S.A.); and isooctane (2,2,4-trimethylpentane), distilled-in-glass grade, from Burdick and Jackson (Muskegon, MI, U.S.A.). Press-fitt connectors were obtained from Hewlett-Packard (Avondale, PA, U.S.A.) and from Restek (Press-Tight™ Connectors, Bellefonte, PA, U.S.A.). The wide-bore, thick-film, FS capillary column (Megabore™, DB-5, 30 m × 0.52 mm I.D., 1.5 μm film thickness) was obtained from J & W (Rancho Cordova, CA, U.S.A.); and the narrow-bore, thin-film, FS capillary column (HP-1, cross-linked methyl silicone, 12 m × 0.2 mm I.D., 0.33 μm film thickness) from Hewlett-

Packard. All other FS capillary columns and the OV-1701-vinyl deactivated pre-columns used in this work were laboratory prepared as previously described [10].

Sample preparation

Standards of D-517, verapamil and norverapamil (hydrochlorides) were prepared in methanol at 40–200 parts per million (μg/ml) and 40–200 parts per billion (ng/ml). The free bases for GC analysis were obtained by placing 2 ml of standard into clean 150 × 16 mm Pyrex screw-cap culture tubes with PTFE cap liners. The methanol was removed with a stream of dry nitrogen under mild heat (43°C), and 2 ml of double-distilled water, 100 μl of concentrated ammonium hydroxide (29.3% NH_3) and 5 ml of isooctane were added. The mixture was vortexed for 2 min, allowed to stand for 10 min, vortexed again and centrifuged for 5 min at 540 *g*. The organic layer (isooctane) was transferred to a 100 × 13 mm Pyrex screw-cap culture tube which had been tapered in a flame. The isooctane was evaporated with a stream of dry nitrogen under mild heat (43°C) and the standards were re-dissolved in 100 ml of isooctane. A portion of this solution was transferred to a 0.1-ml automatic injector vial for analysis by cold on-column injection capillary GC using the automatic sampler.

Gas chromatography

Capillary GC was performed with a Hewlett-Packard 5890A gas chromatograph equipped with a dedicated on-column capillary inlet, a flame ionization detection (FID) system, a NPD system and a 7693A automatic sampler. The GC and automatic sampler were controlled with a Hewlett-Packard 3393A computing integrator. The FS capillary GC columns and their dimensions used in this work included the following: (1) SE-54, 30 m × 0.32 mm I.D., 0.1 μm film thickness; (2) SE-54, 20 m × 0.52 mm I.D., 0.33 μm film thickness; (3) SE-54,30 m × 0.32 mm I.D., 0.2 μm film thickness; (4) HP-1 (cross-linked methyl silicone), 12 m × 0.2 mm I.D., 0.33 μm film thickness; (5) DB-5, 30 m × 0.52 mm I.D., 1.5 μm film thickness. The temperature programs varied depending on which column was used, but always began at 90°C with a 1 min hold followed by a temperature ramp to the final temperature. The final temperature also varied with the column used (see legends of individual figures for more details). All sample injections were 1 μl of isooctane using the Hewlett-Packard 7693 automatic sampler in the on-column injection mode. In the on-column mode, the automatic sampler uses a syringe equipped with a 26-gauge needle to allow insertion of the needle into 0.52 mm I.D. or larger columns. All attachment of pre-columns to the various FS capillary columns were done with the press-fit connectors. When using NPD, the detector attenuation was initially set at low sensitivity (attenuation = 5–8) and automatically changed to high sensitivity (attenuation = 0–1) just before elution of the standard components.

RESULTS AND DISCUSSION

Evaluation of the press-fit connectors at high sample (μg/ml) levels was accomplished by analysis of our standard mixture (Fig. 2A) using a wide-bore FS capillary column, then removing a 1-m section of the column and re-connecting it as a pre-column using a press-fit connector, and again analyzing the standard mixture

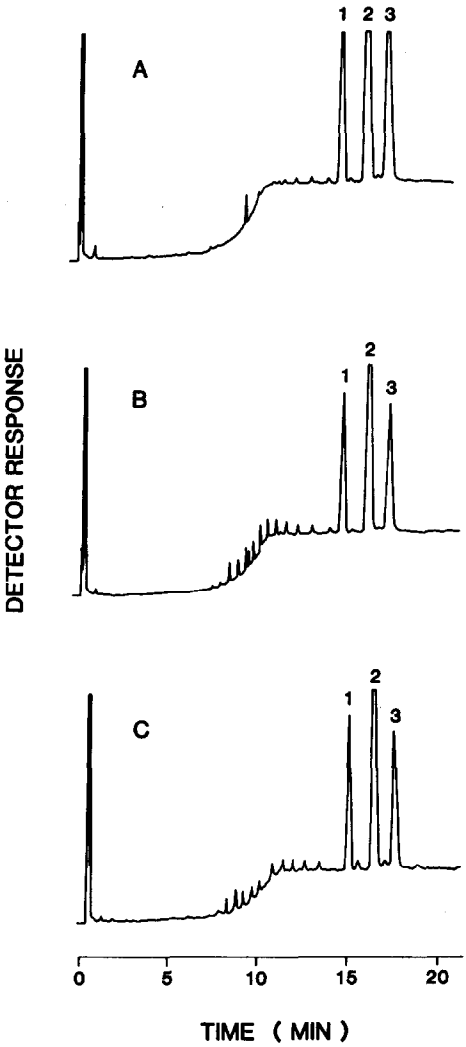

Fig. 2. Cold on-column injection capillary GC separation of a standard mixture of (1) D-517 (IS), (2) verapamil and (3) norverapamil using a wide-bore FS SE-54 column. Conditions: column dimensions, 20 m × 0.52 mm I.D.; film thickness, 0.3 μm; temperature program, 90°C for 1 min, 90–280°C at 10°C/min; column flow, 60 cm/s helium; detection, FID; FID temperature, 330°C. (A) Column only, (B) column with 1 m removed and reconnected as a pre-column using a press-fit connector, and (C) column only with 0.01% TEA added to the sample.

(Fig. 2C). We also evaluated a basic chemical modifier, triethylamine (TEA, 0.01%), added to the solvent (isooctane), to change and/or improve the results (Fig. 2B) [8]. The precision (% relative standard deviation) of the response factors [K = (areas x/amount x)/(area IS/amount IS)] for verapamil and norverapamil were 0.2 and 1.7%, respectively, for the three analyses in Fig. 2 based on the IS, D-517. Thus, at these levels (μg/ml), the press-fit connector appears to have no effect upon the analysis of verapamil and norverapamil, nor does the addition of TEA.

We also found that the attachment of a 1-m pre-column of 0.52 mm I.D. FS tubing deactivated with OV-1701-vinyl [10] had no effect on the analysis at μg/ml levels. The effect of the length of the pre-column was studied for several lengths between 0.07 and 3.00 m. Analysis of our three components at μg/ml levels using a 0.07-m pre-column (Fig. 3A) and a 3.00-m pre-column (Fig. 3B) showed no effect from the difference in pre-column length.

The classic works on pre-columns or "'retention gaps" [1] showed that these initial column sections of very low retention efficiently eliminated band broadening in space. We also looked at the effects of the film thickness of the pre-columns (Fig. 4). The differences in chemical reactivity of verapamil and its active metabolite, norverapamil, are clearly illustrated by the chromatogram obtained using the column with a blank (undeactivated) FS pre-column (Fig. 4A). Although the verapamil peak is broadened, its response relative to the IS (D-517) was unaffected, while norverapamil was completely absorbed by the blank FS pre-column. As expected, a pre-column deactivated with OV-1701-vinyl (Fig. 4B) gave good results, but a pre-column with a 0.33-μm film of SE-54 resulted in peak broadening and splitting (Fig. 4C). A pre-column with higher retention power than the capillary column need not be very long to produce these peak distortions. A 0.07-m FS pre-column with a 0.33-μm film of

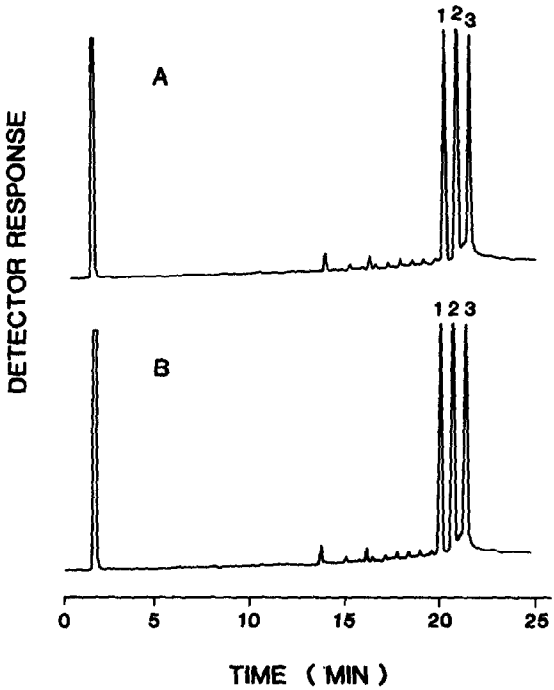

Fig. 3. Cold on-column injection capillary GC separation of a standard mixture of (1) D-517 (IS), (2) verapamil and (3) norverapamil using a FS SE-54 column. Conditions: column dimensions, 30 m × 0.32 mm I.D.; film thickness, 0.1 μm; temperature program, 90°C for 1 min, 90–280°C at 10°C/min; column flow 43 cm/s helium; detection, FID; FID temperature, 330°C. (A) Column with a deactivated (OV-1701-vinyl) 0.07 m × 0.52 mm I.D. FS pre-column, (B) column with a deactivated (OV-1701-vinyl) 3.00 m × 0.52 mm I.D. FS pre-column.

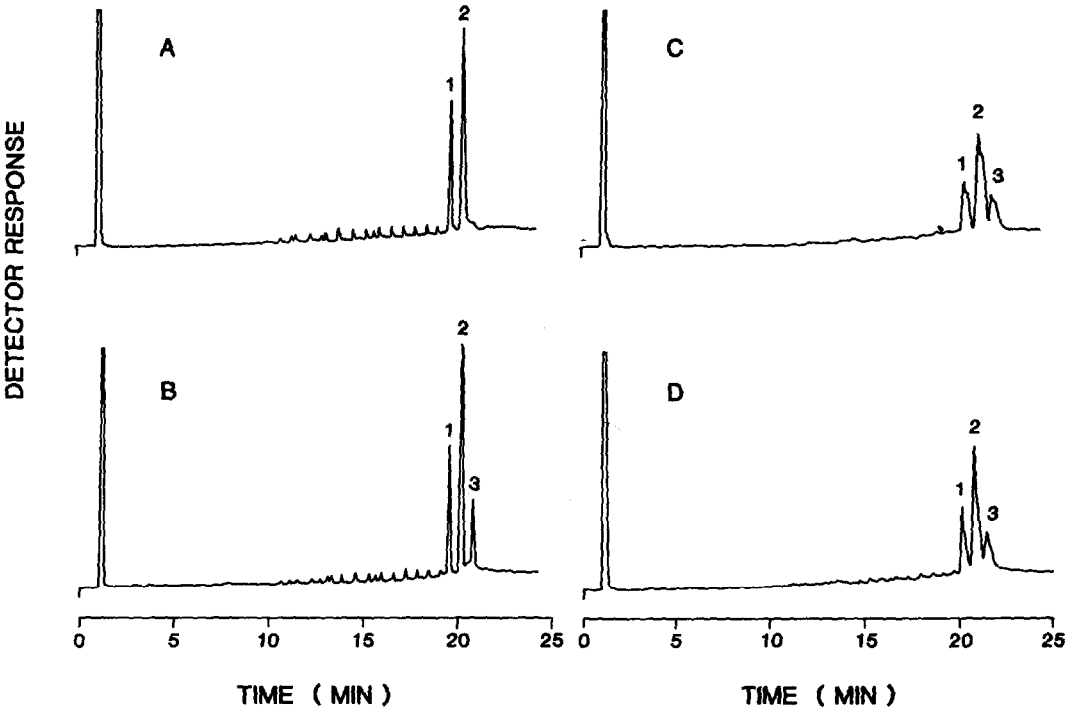

Fig. 4. Cold on-column injection capillary GC separations of a standard mixture of (1) D-517 (IS), (2) verapamil and (3) norverapamil using a 30 m × 0.32 mm I.D. FS SE-54 column with a 0.1 μm film thickness. Conditions: see Fig. 3. (A) Column with a 0.1 μm film thickness. Blank (undeactivated) 0.5 m × 0.52 mm I.D. FS pre-column, (B) column with a deactivated (OV-1701-vinyl) 0.5 m × 0.52 mm I.D. FS pre-column, (C) column with a 0.5 m × 0.52 mm I.D. FS pre-column which had a 0.33-μm film of SE-54 and (D) column with a 0.07 m × 0.52 mm I.D. FS pre-column which had a 0.33-μm film of SE-54.

SE-54 also results in peak splitting (Fig. 4D). The addition of TEA to the solvent (0.01%) did not affect the results.

One means of reducing the number of active sites in a column, or in this case a pre-column, is the repeated injection of large amounts of the species of interest. Separation of the standard mixture at μg/ml levels using a blank (undeactivated) pre-column produced the expected absorption of norverapamil (Fig. 5A). Injection of a concentrated solution of norverapamil (Fig. 5B) yielded a small, broad peak. Analysis of the standard mixture (Fig. 5C) showed that the activity of the pre-column was reduced as a peak for norverapamil was present although its height was less than expected. The second injection of the concentrated solution of norverapamil (Fig. 5D) yielded a much larger peak, but repeated injection of the standard mixture (Fig. 5E) showed little change. Injection of the μg/ml solution of norverapamil using a column with a 1-m deactivated (OV-1701-vinyl) pre-column (Fig. 5F) produced a large peak indicating that absorption of norverapamil even at the relatively high μg/ml levels was still occurring in the undeactivated pre-columns (Fig. 5B and D). The response (peak area) of verapamil in comparison to the IS (D-517) remained constant regardless of the pre-column used (Fig. 5).

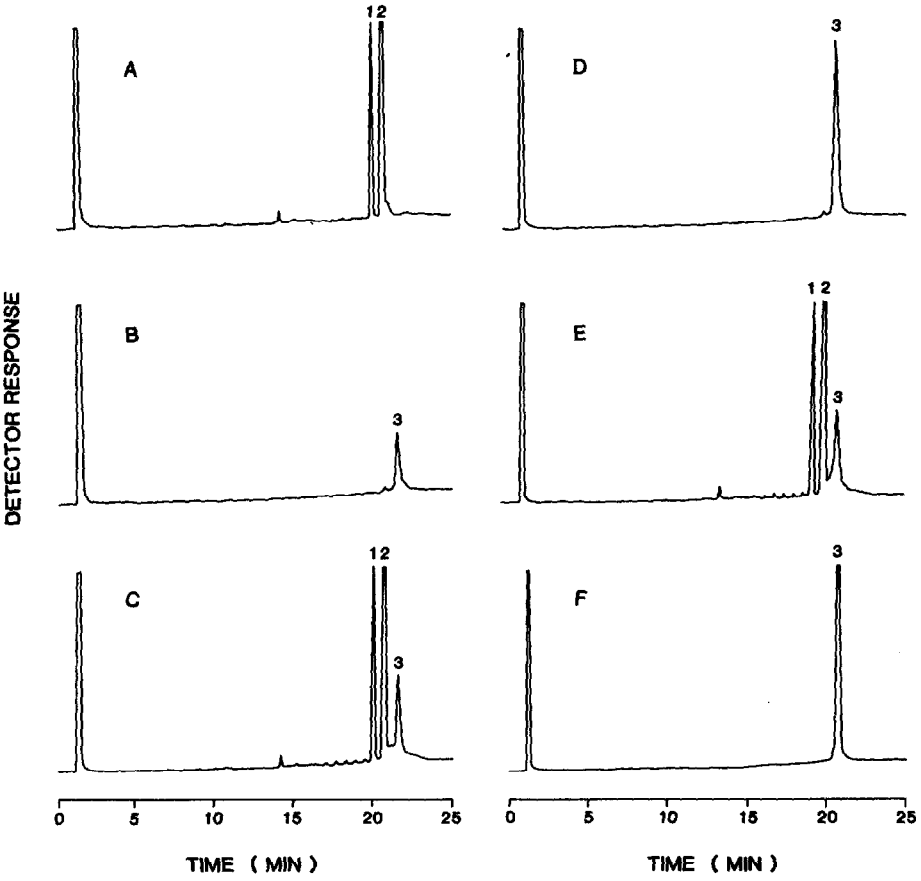

Fig. 5. Cold on-column injection capillary GC separations of (1) D-517 (IS), (2) verapamil and (3) norverapamil using a FS SE-54 column. Conditions as in Fig. 3. (A) Analysis of all three components using a column with a blank (undeactivated) 1 m × 0.52 mm I.D. FS pre-column, (B) analysis of a concentrated sample of norverapamil again using the column and blank pre-column, (C) analysis of the three-component mixture after injection of the concentrated norverapamil sample also using the column with blank pre-column, (D) second separation of the concentrated norverapamil sample, (E) third analysis of the three-component mixture and (F) separation of the concentrated verapamil sample using the column with a deactivated (OV-1701-vinyl) 1 m × 0.52 mm I.D. FS pre-column.

The effects of the deactivated (OV-1701-vinyl) pre-column and the press-fit connector on the GC analysis at low levels (ng/ml)) were investigated using a wide-bore, thick-film, FS capillary column with NPD (Fig. 6). First, our concentrated mixture (μg/ml) was chromatographed using FID (Fig. 6A). Next, the pre-column was removed and the ng/ml-level standard mixture was separated using the column without a pre-column, using NPD (Fig. 6B). Finally, the deactivated pre-column was re-connected and the sample (ng/ml) was chromatographed once again. Results were the same with or without the pre-column (Fig. 6B and C) indicating that the deactivated (OV-1701-vinyl) pre-column and the press-fit connector did not contribute to peak absorption, tailing, or broadening, especially in the case of the more reactive

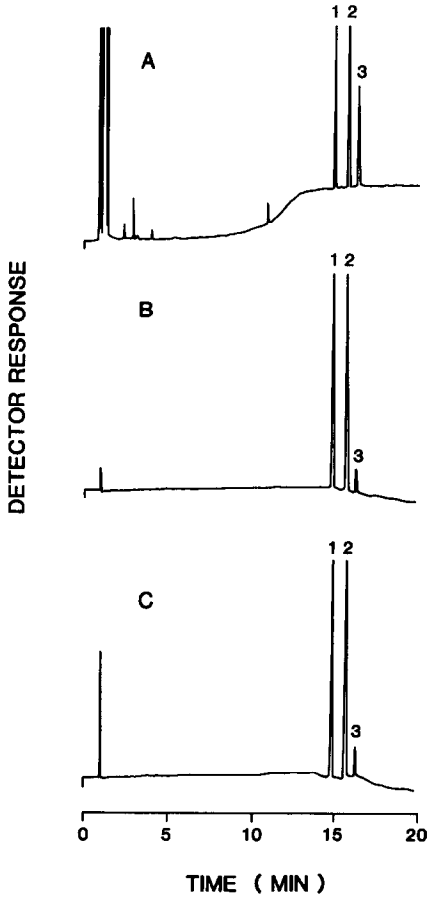

Fig. 6. Separation of (1) D-517 (IS), (2) verapamil and (3) norverapamil with a wide-bore, thick-film, FS DB-5 column at high (μg/ml) concentrations using FID and at low, therapeutic (ng/ml) levels using NPD. Conditions: column dimensions, 30 m × 0.52 mm I.D.; film thickness, 1.5 μm; column flow, 63 cm/s helium; temperature program, 90°C for 1 min, 90–310°C at 20°C/min; FID temperature, 330°C; NPD temperature 280°C. (A) Separation of concentrated (μg/ml) mixture using FID and a deactivated 0.75 m × 0.52 mm I.D. FS pre-column, (B) separation of the low-level (ng/ml) mixture using NPD and the column only (no pre-column) and (C) separation of the low-level (ng/ml) mixture using NPD and the column with a deactivated 0.75 m × 0.52 mm I.D. FS pre-column.

norverapamil. The response factors (K) for verapamil in relation to the IS (D-517) were constant even when those at high levels (μg/ml) (Fig. 6A) were compared to those obtained at low, therapeutic levels (ng/ml) (Fig. 6B and C) using two different detector systems (FID and NPD). However, the response factors (K) of norverapamil were 5 × less at ng/ml levels using NPD compared to those at μg/ml levels using FID. Some change in response was anticipated due to the difference in the detector systems, but the magnitude (5 ×) of that change was surprising. Although the peak shapes were unaffected (Fig. 6B and C), the huge unexplained difference in response could

result from absorption of the relatively reactive norverapamil at ng/ml levels even using a very-low-surface-activity, commercially produced column.

The repeated injection of large amounts of norverapamil to improve its response was studied at therapeutic levels (ng/ml) using a 30 m × 0.32 mm I.D. FS SE-54 column with a 0.25 μm film thickness and a 1-m deactivated pre-column (Fig. 7). Initial separation of the standard mixture (Fig. 7A) was almost identical to the results obtained after repeated injection of a concentrated solution of norverapamil (Fig. 7B).

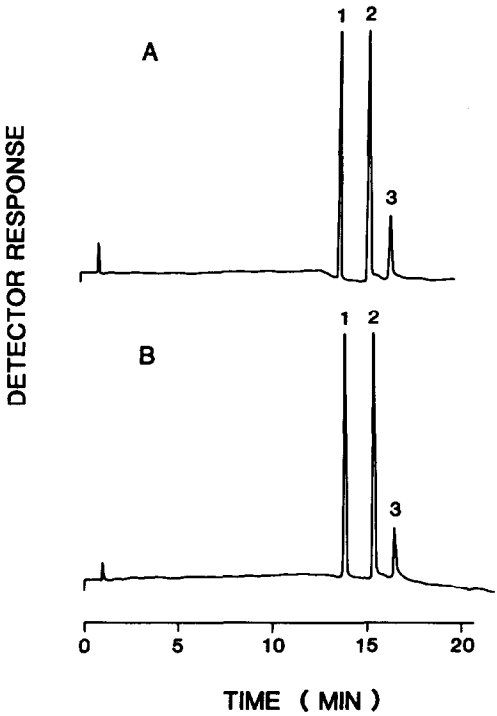

Fig. 7. Separation of (1) D-517 (IS), (2) verapamil and (3) norverapamil at therapeutic (ng/ml) levels using a FS SE-54 column. Conditions: column dimensions, 30 × 0.32 mm I.D.; film thickness, 0.25 μm; column flow, 45 cm/s helium; temperature program, 90°C for 1 min, 90–280°C at 20°C/min; detection, NPD; NPD temperature, 280°C; pre-column, 1 m × 0.52 mm I.D. deactivated (OV-1701-vinyl) FS. (A) Analysis of standard three-component mixture and (B) analysis of mixture after several injections of a concentrated sample of norverapamil.

The analysis of the three-component mixture at μg/ml and ng/ml levels using a 12 m × 0.2 mm I.D. FS methylsilicone (HP-1) capillary column with a 0.3-m deactivated pre-column is shown in Fig. 8. Peaks shapes are not as good at low levels (Fig. 8B and C), and the response of norverapamil decreases by over 50% after only 12 repeated analyses (Fig. 8C), while the response of verapamil remained constant.

Using a wide-bore FS SE-54 column, we again demonstrated that analysis of D-517 (IS), verapamil and the more reactive norverapamil at therapeutic (ng/ml) levels was unaffected by the attachment of a 1-m deactivated (OV-1701-vinyl) FS

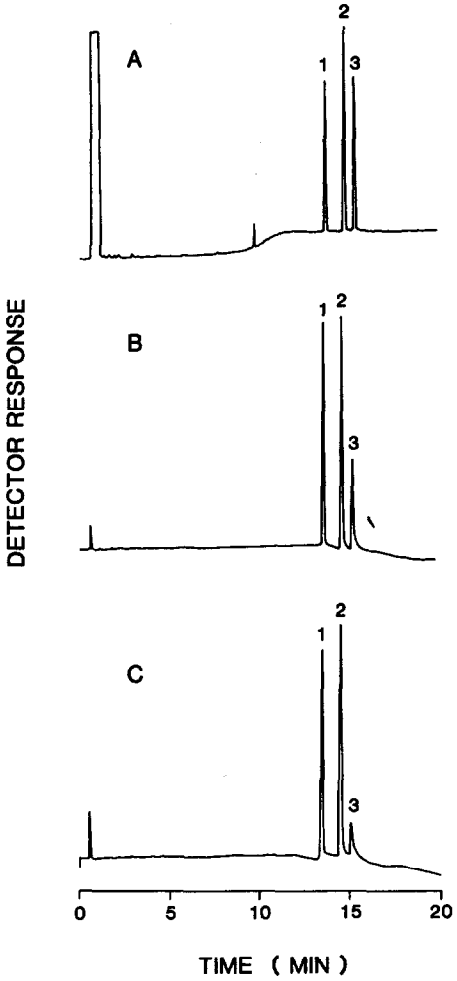

Fig. 8. Separation of (1) D-517 (IS), (2) verapamil and (3) norverapamil, using a narrow-bore, methyl silicone (HP-1) FS column with a 0.3 m × 0.52 mm I.D. deactivated (OV-1701-vinyl) FS pre-column, at high (μg/ml) levels using FID and at therapeutic (ng/ml) levels using NPD. Conditions: column dimensions, 12 m × 0.2 mm I.D.; film thickness, 0.33 μm; column flow, 30 cm/s helium; temperature program, 90°C for 1 min, 90–280°C at 20°C/min; FID temperature, 330°C; NPD temperature, 280°C. (A) Separation of concentrated (μg/ml) mixture using FID, (B) separation of the low-level (ng/ml) mixture using NPD and (C) separation of the low-level mixture after 12 repeated analyses.

pre-column using a press-fit connector (Fig. 9A and B). A blank undeactivated pre-column had little or no effect on D-517 and verapamil, but norverapamil was again completely absorbed (Fig. 9C). This was in sharp contrast to the appearance of the chromatogram showing the separation of a concentrated sample of norverapamil also using the blank undeactivated pre-column (Fig. 9D). The well-defined, symmetrical peak at higher ng/ml levels belies the complete absorption of norverapamil at therapeutic concentrations.

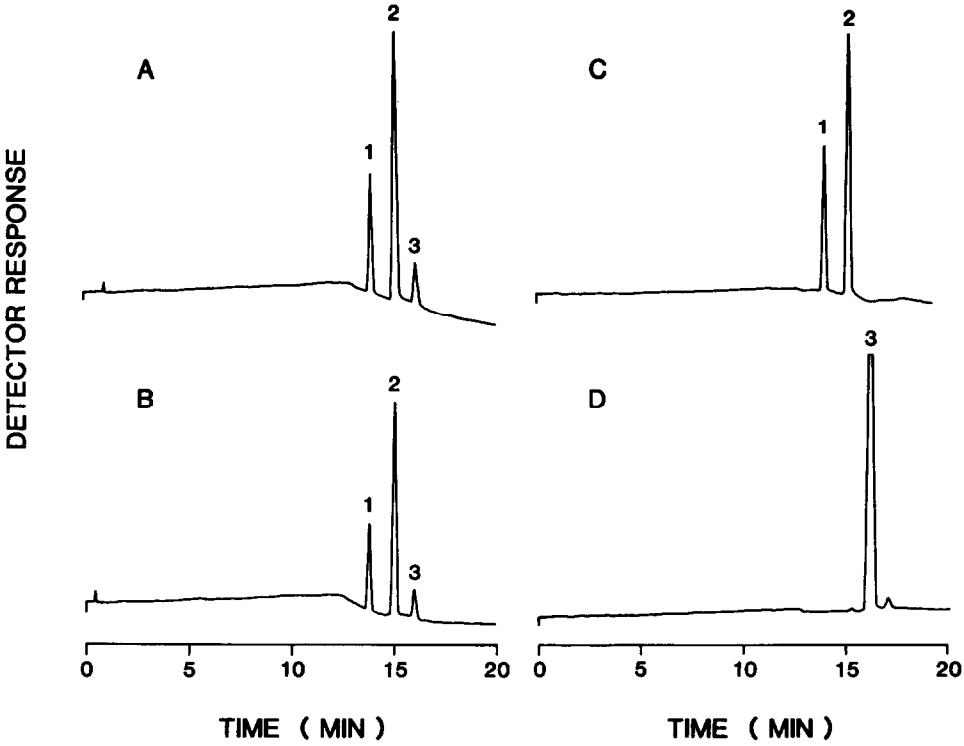

Fig. 9. Separation of (1) D-517 (IS), (2) verapamil and (3) norverapamil using a wide-bore FS SE-54 column. Conditions: column dimensions, 20 m × 0.52 mm I.D.; film thickness, 0.25 μm; column flow, 66 cm/s helium; temperature program, 90°C for 1 min, 90–280°C at 20°C/min; detection, NPD; NPD temperature, 280°C. (A) Column only, (B) column with a 1 m × 0.52 mm I.D. deactivated FS pre-column; (C) column with a 1 m × 0.52 mm I.D. blank pre-column and (D) a concentrated sample of norverapamil separated on the column with a blank pre-column.

CONCLUSIONS

The relatively new press-fit connectors are an excellent means of attaching pre-columns to coated capillary columns. They did not significantly increase the activity of our capillary columns even at ng/ml levels. Their insertion into columns had no detectable effects even on the analysis of the more reactive norverapamil. Deactivation with OV-1701-vinyl was an acceptable means of preparing FS tubing for pre-columns as their use had no effect upon the analysis of D-517, verapamil and norverapamil at therapeutic (ng/ml) concentrations. The addition of TEA to the samples or the repeated injection of large amounts of norverapamil were ineffective in the elimination of surface activity of a column and/or pre-column. The active metabolite norverapamil exhibited much higher reactivity toward active sites in chromatographic systems than verpamil, and detector response for norverapamil increased as the amount injected increased, while the response of verapamil remained constant. In addition, repeated analyses of norverapamil at ng/ml levels yielded a decrease in response over time using both commercially and laboratory-prepared columns with

and without deactivated pre-columns. Thus, conditions which are acceptable for the analysis of verapamil may be unacceptable for the determination of norverapamil. These data indicate that the reliable analysis of norverapamil by GC requires that it first be converted to a more stable derivative. However, the attachment of a deactivated pre-column had no effect on the analysis of verapamil or norverapamil.

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