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AUTOMATED ANALYSIS OF ANTIEPILEPTIC DRUGS IN SERUM BY COLUMN-SWITCHING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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SUMMARY

An automated high-performance liquid chromatographic column-switching system is presented for the analysis of antiepileptic drugs in serum. Initial results show that a reversed-phase extraction column works best overall when fitted with screens versus frits, and when packed with porous 30-μm particles as opposed to a pellicular packing of similar size or with smaller porous particles. The continuous analysis of primidone for over 2000 serum samples is achieved at a rate of twelve samples per hour with a single analytical column. An analogous boxcar high-performance liquid chromatographic system is also assembled and used to analyze two of four injected antiepileptic drugs at a rate of 40 samples per hour. For 1000 of these analyses, the coefficient of variation is 1% without an internal standard.

INTRODUCTION

The use of column-switching techniques for automated sample cleanup in high-performance liquid chromatography (HPLC) has been recently reviewed [1] with articles continuing to appear on this general method [2—8]. The

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major significance of this approach is that sample pretreatment has often been the rate-limiting step in analysis by HPLC.

In this article we extend column-switching HPLC to the analysis of antiepileptic drugs in serum. After first investigating the characteristics of particle size and type, screen versus frit end-fittings, and washing conditions in a reversed-phase extraction precolumn, we then illustrate a fully automated system that maintains high performance throughout the continuous analysis of over 2000 serum injections. We also demonstrate the capability and enhanced throughput of a boxcar configuration for our automated system.

EXPERIMENTAL

Apparatus

The fully automated HPLC system shown in Fig. 1 was constructed from a dual-piston Model 100 pump (Altex, Berkeley, CA, U.S.A.); a Model M 6000 HPLC pump (Waters Assoc., Milford, MA, U.S.A.); an autosampler and a peristaltic pump (Technicon Instruments, Tarrytown, NY, U.S.A.); two Model CV-6-UHPa-N60 pneumatically driven (nitrogen) six-port switching valves (Valco Instruments, Houston, TX, U.S.A.); and a Model 7010 sample injection valve (Rheodyne, Cotati, CA, U.S.A.), pneumatically driven with helium as described in the text. The injection valves and the autosampler were controlled by a Micromaster Model WP 6001 digital programmable timer (Minarik Electric, Los Angeles, CA, U.S.A.). Detection was carried out at 214 nm with a UV III spectrometer equipped with zinc source supply (Laboratory Data Control, Riviera Beach, FL, U.S.A.), using a dual-pen recorder (Linear Instruments, Reno, NV, U.S.A.).

The chromatographic system with boxcar capability involved two Model 110 A high-pressure pumps (Altex), a Series E-120 low-pressure pump (Eldex Labs., Menlo Park, CA, U.S.A.); a FAST-LC variable-wavelength photometric detector, equipped with a 12-μl flow cell (Technicon Instruments); and a SP-4100 computing integrator (Spectra-Physics, San Jose, CA, U.S.A.). The remaining components were the same as for the above system.

Stationary phases and columns

The analytical column was a FAST LC-8 column (150 × 4.6 mm) packed with 5-μm particles of porous silica bonded with dimethyl octylchlorosilane (Technicon Instruments); the short column was a 50 × 4.6 mm column packed with 5-μm particles of C_8 bonded phase packing material (Supelco, Bellefonte, PA, U.S.A.); and the extraction column, unless noted otherwise, was a 25 × 3.9 mm guard column (Waters Assoc.) dry-packed with 30-μm reversed-phase particles, LiChrosorb Si 60 (Supelco) bonded with C_{18} in our laboratory.

Other stationary phases employed for dry packing the extraction column included C_{18} Corasil®, 37—50 μm particle size (Waters Assoc.); LiChrosorb RP-18, 10 μm particle size (Alltech Assoc., Deerfield, IL, U.S.A.); and Supelcosil C_8, 5-μm particles (Supelco).

Reagents

Primidone and phenytoin were obtained from Applied Science Labs.

(Deerfield, IL, U.S.A.). Phenobarbital and carbamazepine were supplied by Technicon Instruments. Stock solutions of the antiepileptic drugs were made in methanol. Dilutions of the stock standards were made with pooled serum as required. Methanol was of chromatographic grade (J.T. Baker, Phillipsburg, NJ, U.S.A.). Sequanal grade triethylamine was purchased from Pierce Chemical (Rockford, IL, U.S.A.). Glass-distilled water was used throughout.

Analytical mobile phase

This reagent was 2.5 m*M* sodium dihydrogen phosphate in 45% methanol and contained 65 μl/l triethylamine. It was passed through a 0.45-μm filter (Millipore, Bedford, MA, U.S.A.) and degassed in vacuo prior to use.

Washing solution

Glass-distilled water degassed in vacuo was used throughout. Buffer was 0.1 *M* sodium monophosphate adjusted to pH 3.5 with phosphoric acid, and filtered as above.

Defibrination of plasma

Outdated, pooled blood bank plasma (1 l) was defibrinated by the addition of 2.22 g of calcium chloride followed by stirring for 1 h with a glass rod. After standing at room temperature for 2 h, the jelly-like mass was broken up with a glass rod and the clear supernatant was filtered through a Whatman filter paper, centrifuged, and frozen overnight. The supernatant was then thawed out at room temperature and filtered before use, giving a clear filtrate. The unused portion was frozen and re-filtered before use.

RESULTS AND DISCUSSION

Basic configuration

The basic configuration of our fully automated HPLC system with column-switching is shown in Fig. 1. Included is a six-port Valco loading valve (V_1) with a sample loop (L) and a six-port Valco injection valve (V_2), with a small,

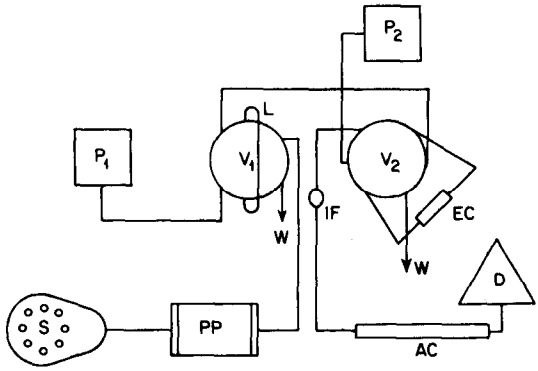

Fig. 1. Configuration of the fully automated HPLC system with on-line, solid-phase sample cleanup. S, auto sampler; PP, peristaltic pump; L, sample loop; V_1 and V_2, six-port switching valves; W, waste; P_2, mobile-phase pump; P_1, wash pump; EC, extraction column; AC, analytical column; IF, in-line filter; and D, detector.

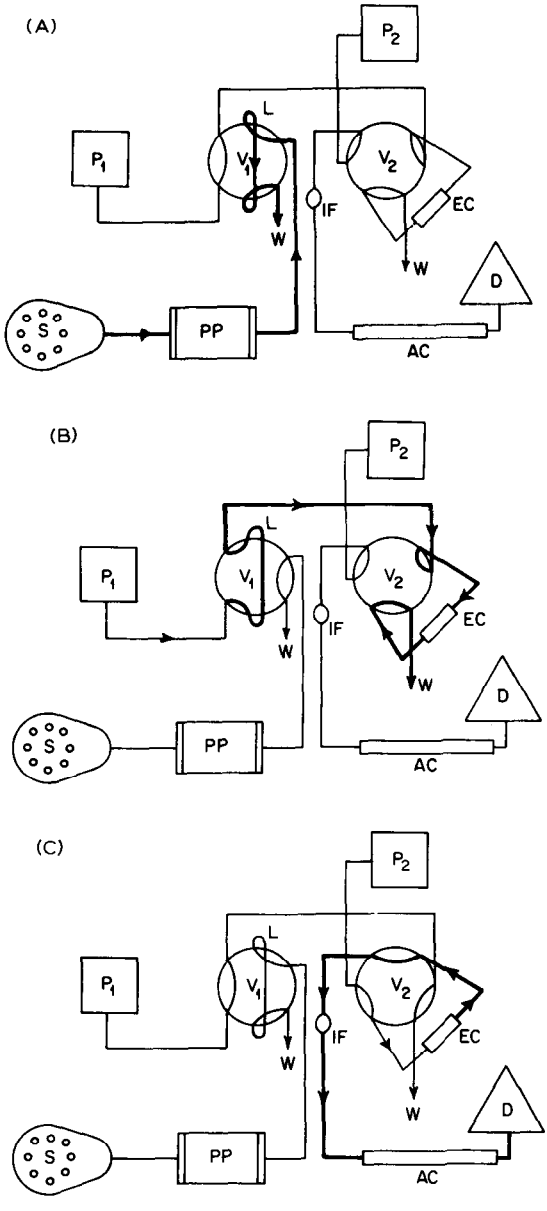

Fig. 2. Working cycle of the automated HPLC system involving solid-phase extraction and column-switching with the sample flow paths as heavy lines. (A) Activiation. The autosampler (S) is activated by the programmable timer to pick up the sample and fill the sample loop (L) by means of the peristaltic pump (PP) with excess going to waste (W). Simultaneously, the extraction column (EC) is equilibrated with flushing solvent (distilled water or buffer, pH 3.5) delivered by wash pump (P_1). (B) Loading. The loading valve (V_1) is rotated and the sample is washed onto the extraction column (EC). Since the extraction column is packed with reversed-phase material and conditioned with aqueous wash, the analyte(s) are adsorbed and enriched at the top of this column while the hydrophilic substances are washed through the extraction column to waste (W). (C) Injection. The injection valve (V_2) is next reversed and the sample components are back-flushed onto the analytical column (AC) for separation and analysis.

dry-packed extraction column (EC) for solid-phase extraction. The analytical column (AC) and the mobile-phase pump (P_2) are connected to V_2 whereas the wash pump (P_1) is connected to the V_1 loading valve, as is the autosampler (S) in series with the peristaltic pump (PP). The autosampler and the two valves are actuated automatically by a programmable timer based on a fixed time schedule. The whole working cycle in the analysis of a typical serum sample consists of an activation step, a loading step and an injection step, as illustrated and described in Fig. 2A—C. The heavy lines represent the flow-paths of the sample.

Extraction column

At the outset of our investigation, we were particularly encouraged by the results of Roth et al. [9] in the use of column-switching as a cleanup tool. These workers had reported on a system that successfully analyzed drugs in directly injected plasma, urine, or saliva samples. The system was based on the use of an extraction precolumn comprised of either reversed-phase or ion-exchange material, having a particle size of about 20—50 μm. After the drug was adsorbed from the sample, the precolumn was washed with water prior to elution of the drug with a backflush of mobile phase onto the analytical column. For a reversed-phase extraction precolumn, at least 1000 analyses could be conducted with the use of two such precolumns operated in parallel without a need for replacing the columns.

We began with a system similar to that reported by Roth et al. [9], having the components and configurations shown in Fig. 1, except for the sample delivery components (autosampler, peristaltic pump), programmable timer, and use of a single precolumn. The sample loop was filled by means of a syringe and the switching valves were actuated manually. The analysis of primidone spiked into serum was selected as an initial application. We considered that success with this highly polar drug, having weak adsorption characteristics for trapping onto a reversed-phase extraction column, would provide conditions also applicable to the other antiepileptic drugs having less polar structure.

We began our studies with an extraction column that was enclosed with 2-μm porous frits, and contained a porous 5-μm bonded-phase silica packing as opposed to much larger particles (Waters C_{18} Corasil) used by Roth et al. [9]. Our results with this approach were quite discouraging. After less than 100 consecutive serum injections of 100 μl each, we observed a severe increase in the pressure drop across both the extraction column and analytical column, and also a major drop in the overall plate number for the primidone peak.

We next packed our extraction column with the same type of material used by Roth et al. [9] and obtained significantly better results. After 75 serum injections of 100 μl each, there was no increase in back pressure across the extraction column, a moderate increase (17.7 bars) across the analytical column, and a decrease of 38% in the overall plate number. These results, however, still seemed to fall short of those reported by Roth et al. [9].

Our next step was to use an extraction column that we conjectured to be closer to that used by Roth et al. [9], comprising a standard, Waters guard column fitted with Waters screens (a retainer screen with 0.2-mm porosity against the particles, backed by a filter screen having 2-μm porosity), and we

repeated the 100 serum injections. This time our results agreeed with those of Roth et al. [9] in that there was no change in back pressure across either the extraction or the analytical column and the overall plate number remained constant. The importance on the use of screens instead of frits to prevent sample blockage has also been recently emphasized by Roth [10].

Based on these results, we decided to re-examine in more detail the role of the particle size and type in the extraction column, employing the Waters screens instead of porous frits as endfittings. The four reversed-phase silica particles that we tested and their performances are summarized in Table I.

TABLE I

PARAMETERS AFFECTED BY VARIATION OF PARTICLE SIZE AND TYPE IN THE EXTRACTION COLUMN

Particle size and type in the extraction column*	Changes in parameter after 100 injections of 100 μl of serum spiked with primidone			
	Increase in pressure across the extraction column (bars)	Increase in pressure across the analytical column (bars)	Initial and final plate numer of the analytical column**	Absolute recovery of primidone (%)
5-μm C_8	13.6	27.2	7080, 2600	100
10-μm C_{18}	3.4	13.6	6305, 3560	100
Corasil (37—50 μm, C_{18})	0	1.36	7400, 7450	5
30-μm C_{18}	6.8	0	8325, 7942	>90

*The extraction column was a 25 × 3.9 mm column fitted with a retainer screen and 2-μm filter screen (Waters Assoc.).

**Plate number was calculated using toluene and the mobile phase of the analytical column.

Although the recovery of primidone with the 5-μm, C_8 porous particles was quantitative throughout 100 injections of 100 μl each of serum spiked to a concentration of 20 μg/ml with this drug, the plate number of the analytical column dropped significantly. Also, a gradual increase in pressure developed across both the extraction and analytical columns. With a 10-μm C_{18} porous packing in the extraction column, the quantitative recovery of primidone was maintained, but the reduction in plate number was still significant. Once again an increased pressure developed in the analytical column, although less than before. As already reported above, we observed no significant change in the pressure drop across either the extraction or analytical columns, nor in the plate number of the analytical column, when a Corasil C_{18} pellicular packing was used. However, now we observed that the recovery of primidone was only about 5% with this packing.

The best overall results were obtained with a porous 30-μm C_{18} packing, as shown in Table I. This column gave a greater than 90% recovery of primidone, essentially no change in either the pressure drop or plate number of the analytical column, and underwent an intermediate increase in pressure across the extraction column. The higher recovery of drug with this packing relative

to the pellicular packing is readily explained by the higher surface area of the porous particle, as opposed to that of the pellicular Corasil packing. (For a less polar drug such as carbamazepine, or the drugs analyzed by Roth et al. [9], the reversed-phase Corasil pellicular packing does give complete recovery in our system.)

The overall poor performance of the 5- and 10-μm packings, including the previously observed similar shortcomings of porous frits versus screens as retainer fittings, is not completely clear. Perhaps the causes relate to plugging from microparticles or other species present in the serum. Nevertheless, we chose to continue using an extraction column fitted with the Waters screens and packed with porous 30-μm C_{18} particles for our subsequent work.

Recovery of other drugs, and water versus buffer wash

The recovery of three other commonly used antiepileptic drugs was next determined on the fully automated system shown in Figs. 1 and 2. Serum samples spiked with known amounts of drugs were injected onto the extraction column via a 20-μl sample loop followed by forward wash with either distilled water or a low-pH buffer at a flow-rate of 2 ml/min. After washing for 4 min, the system was switched to backflush mode. When distilled water was used as a flushing solvent, high recoveries were obtained in all cases, as shown in Table II, except for phenobarbital. Apparently this latter drug, having a pK_a of 7.3 [11], was ionized and lost during the wash cycle with water. This ionization was suppressed by the acidic wash buffer, yielding a good recovery for phenobarbital and the other drugs as well. A representative chromatogram is shown in Fig. 3.

TABLE II

DRUG RECOVERY FROM SERUM BY AUTOMATED SOLID-PHASE EXTRACTION AS A FUNCTION OF WATER VERSUS BUFFER WASH

The drugs were dissolved together in serum at a concentration of 10—20 μg/ml, based on weight, and this solution was analyzed by the system shown in Fig. 1 using a 20-μl sample injection.

Drug	Percentage recovery*	
	Water wash	Buffer wash**
Primidone	89	91
Phenobarbital	0	91
Phenytoin	81	95
Carbamazepine	98	95

*Average, absolute recoveries were determined based on ten injections.
**0.1 *M* Sodium phosphate buffer, pH 3.5.

Although the data are not shown, we also examined the influence of protein binding on drug recovery. This study was done by comparing the recovery of these same drugs spiked to the same concentration in water versus serum. Identical recoveries were obtained, demonstrating that protein binding (ranging from 0% to 10% for primidone, to 65—85% for carbamazepine [12]) had no overall effect on drug capture by our extraction column.

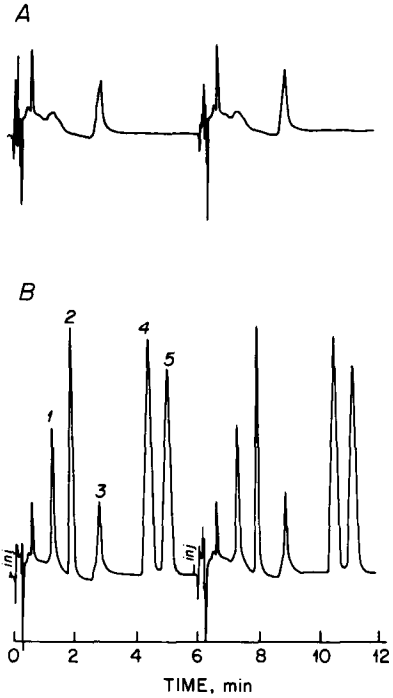

Fig. 3. Representative HPLC chromatograms of pooled serum samples using the systems in Figs. 1 and 2; (A) 20 μl of blank serum pool; (B) 20 μl of serum spiked with primidone (10 μg/ml), phenobarbital (20 μg/ml), phenytoin (15 μg/ml) and carbamazepine (10 μg/ml). A buffer wash (pH 3.5) was delivered by pump P_1. Peaks: 1 = primidone, 2 = phenobarbital, 3 = impurity, 4 = phenytoin, 5 = carbamazepine.

Valve and timing optimization

Encouraged by these results, as summarized in Tables I and II, we introduced further optimization into the system of Figs. 1 and 2 in two respects. First, we reduced the switching time of the injection valve, V_2, to minimize pressure surges on the analytical column, by replacing a Valco Model CV-6-UHPa-N60 with a Rheodyne valve (Model 7010) fitted with a Model 7001 solenoid (Rheodyne). We operated the solenoid with helium in conjunction with a Humphrey TAC[2] 41PP valve. Secondly, the sequential steps of activation, loading and injection were synchronized so that the system handled twelve serum samples per hour. This was accomplished primarily by simultaneously conducting sample pickup into the injection loop (40 sec) and water wash of the extraction column (40 sec), while having these events begin 50 sec after the previous sample had been injected onto the analytical column.

Over 2000 injections

With the optimized version for the system shown in Figs. 1 and 2, as summarized in Table III, we achieved 2112 continuous 20-μl serum injections spiked with primidone with practically no change in the analytical column back pressure. The chromatographic column lost less than 25% of its starting plate number. The extraction column was repacked and its filter replaced whenever its back pressure increased more than 6.8 bars, which typically occurred every

TABLE III

PRESSURE DROP AND EFFICIENCY OF THE ANALYTICAL COLUMN DURING OVER 2000 SERUM INJECTIONS USING THE SYSTEM SHOWN IN FIG. 1

Number of injections	Pressure drop across the analytical column (bars)	Plate number of the analytical column*
0	123.8	8400
146	125.8	8150
249	129.2	8580
462	127.2	8680
760	125.8	7360
1090	125.8	7940**
1261	122.4	6740
1393	129.2	7120
1637	129.2	7390
1877	141.4	6240
2112	144.2	6500

*Evaluated as described in Table I.
**The analytical column was reversed.

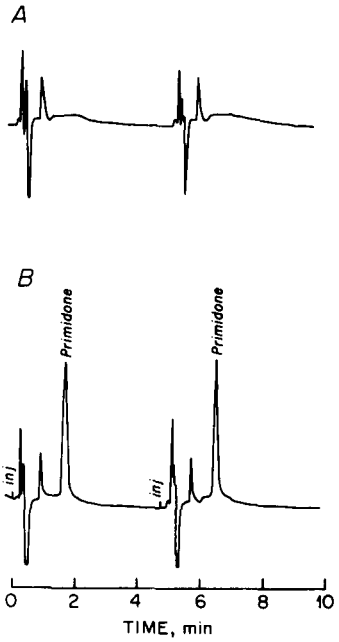

Fig. 4. Typical chromatograms using the automated HPLC system shown in Figs. 1 and 2: (A) 20 μl of serum blank; (B) 20 μl of serum spiked with 10 μg/ml of primidone. A water wash was delivered by pump P_1.

200—300 serum injections. The analytical column was periodically washed with tetrahydrofuran to remove any strongly retained components, and was reversed once during the overall experiment. The recoveries and precision obtained day-to-day with different extraction columns were consistent, as was the absence throughout of any baseline disturbance or additional peaks. The within-day

precision was quite high, ranging from 0.8% to 2.5%, coefficient of variation (C.V.). A representative chromatogram is shown in Fig. 4.

Boxcar configuration

Boxcar chromatography is an advanced column-switching technique placing multiple samples simultaneously into an analytical column, for enhanced sample throughput [13]. To demonstrate this increased throughput capability for our system, we converted it to a boxcar configuration, as shown in Fig. 5. This latter configuration differs from that shown in Fig. 1 by the addition of another pump (P_3), a six-port valve (V_3) and a short column (SC). These new components provide an initial separation and heart-cutting of the sample.

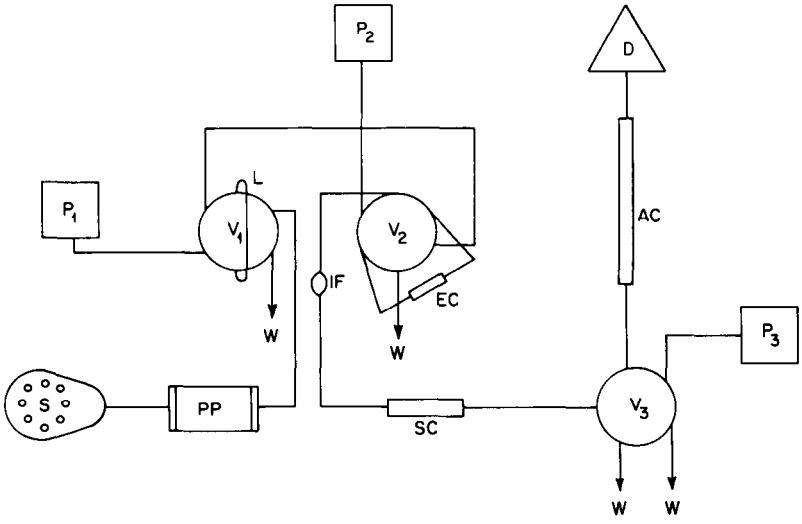

Fig. 5. Configuration of the boxcar HPLC system. S, autosampler; PP peristaltic pump; L, sample loop; V_1, V_2 and V_3, six-port switching valves; W, waste; P_2 and P_3, mobile-phase pumps; P_1, wash pump; EC, extraction column; SC, short column; AC, analytical column; IF, in-line filter; D, detector. The sequential steps for this system, controlled by a digital programmable timer, are as follows: (1) activate the auto sampler, S, to fill up the sample loop, L, while equilibrating the extraction column (EC) with aqueous wash; (2) switch valve V_1 to solid-phase extract the drug in EC; (3) switch valve V_2 to back-flush the drug onto the short column (SC); (4) switch valve V_3 to heart-cut the drug(s) into the analytical column, AC, for further separation prior to detection, D.

With this boxcar system we successfully analyzed primidone and phenobarbital in serum samples containing a mixture of primidone, phenobarbital, phenytoin and carbamazepine at an increased rate of 40 samples per hour. Over 1000 serum samples were analyzed with a C.V. of 1%, without the use of an internal standard (data not shown). A representative chromatogram is shown in Fig. 6.

The timing for this analysis was as follows: (1) sample loading, 20 sec; (2) sample extraction, 48 sec; (3) back elution, 20 sec; (4) pre-separation on short column, 32 sec; and (5) heart cutting onto the analytical column, 30 sec. This timing achieved complete filling of the sample loop; no memory effects from a previous sample; and 100% transfer of the analyte drugs from the short column to the analytical column.

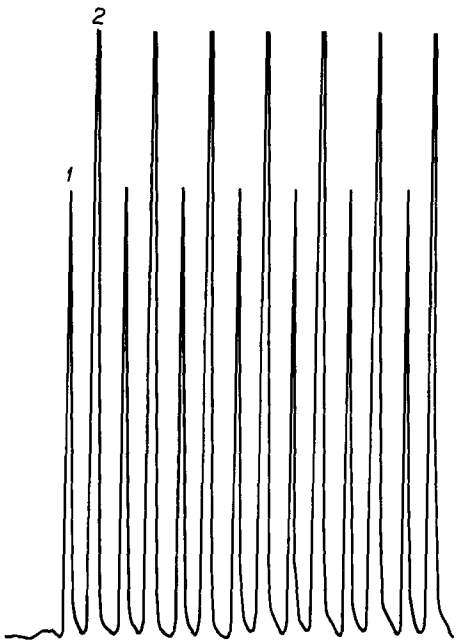

Fig. 6. Chromatogram showing the analysis of primidone (1) and phenobarbital (2) in serum at a rate of 40 samples per hour using the boxcar system shown in Fig. 5.

Aside from the use of parallel columns, further increases in sample throughput utilizing boxcar chromatography potentially may be achieved by additional optimization of the extraction column, since the use of this column currently is the slowest step.

CONCLUSION

Clearly, automated column-switching HPLC is a viable technique for therapeutic drug monitoring. At least for the repetitive analysis of a pooled serum spiked with antiepileptic drugs, we have achieved high throughput, excellent precision, and long-term system stability.

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