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Optimization of multidimensional high-performance liquid chromatography for the determination of drugs in plasma by direct injection, micellar cleanup and photodiode array detection

JOSEPH V. POSLUSZNY* and ROBERT WEINBERGER
Applied Biosystems, Inc., 170 Williams Drive, Ramsey, NJ 07446 (U.S.A.)
and
ERIC WOOLF
Berlex Laboratories, 110 East Hanover Avenue, Cedar Knolls, NJ 07927 (U.S.A.)

ABSTRACT

Improvements in a multidimensional liquid chromatography system for the direct determination of drug substances in blood plasma are reported. The system employs an on-line micellar chromatographic cleanup followed by a reversed-phase analytical separation. The limit of detection of propanolol is improved by a factor 10 compared to previously reported work. The technique is applied towards the determination of a multicomponent mixture of tricyclic anti-depressants in blood plasma. A protocol for optimization is described.

INTRODUCTION

Direct injection of biological fluids into the liquid chromatograph is advantageous from the standpoints of speed, cost, safety, sample identification and analytical recovery. This subject was recently reviewed[1]. The problems encountered here generally include shortened column life, lack of trace enrichment and interferences, both exogenous and endogenous. Many of these problems are addressed by column switching[2–10]. More recently, the use of micellar reagents for the first dimension of the column switching procedure has been shown to have certain advantages[11–12]. These advantages include prolongation of column life through solubilization of proteins, extraction of protein-bound drugs and the ability to finely control the elution pattern on the cleanup column.

The advantages of this micellar–reversed-phase system are exploited and applied to the determination of a multicomponent mixture of a structurally related series of anti-depressant drugs. Guidelines for the selection and optimization of the cleanup or extraction column are provided. A high-sensitivity photodiode array de-

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tector is shown to enhance methods development as well as to be capable of trace analysis (ng/ml) of drugs in biological fluids.

The routine use of this direct sample injection (DSI) technology is demonstrated by the analysis of 450 samples over a three-week time frame. Data are presented for precision and accuracy among days. Fifty assays per day were typical for determining propranolol in blood plasma.

EXPERIMENTAL

Apparatus

The high-performance liquid chromatography (HPLC) and related column switching equipment was described in ref. 11. The system was modified with a second low-pressure solenoid valve, to facilitate solvent switching, as indicated in Fig. 1. In addition, an Applied Biosystems (ABI; Ramsey, NJ, U.S.A.) 1000S Photodiode array detector was employed for multiwavelength chromatographic detection and spectral acquisition.

Reagents and columns

The tricyclic anti-depressant drugs, imipramine (IMI), desipramine (DES), amitriptyline (AMI), and nortriptyline (NOR) were obtained from Sigma (St. Louis, MO, U.S.A.). Doxepin was obtained as a 1-mg/ml solution in methanol from Supelco (Bellfonte, PA, U.S.A.). Sequanal-grade sodium dodecyl sulfate (SDS) was obtained from Pierce (Rockford, IL, U.S.A.). All other reagents and solvents were from sources described in ref. 11. Precise reagent compositions are given in individual figure captions as appropriate.

All of the chromatographic columns were obtained from ABI (formerly Brownlee Labs.).

Detection

Propranolol was measured with fluorescence by using an ABI 980 fluorescence

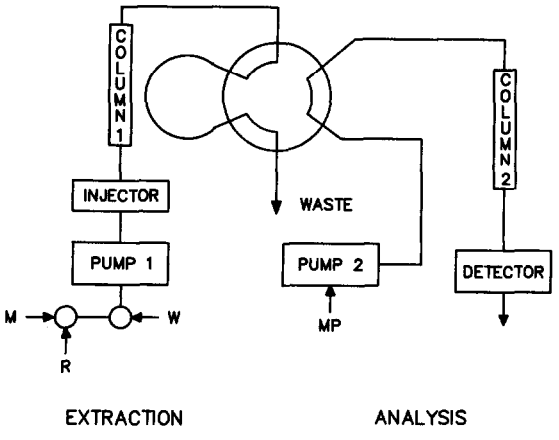

Fig. 1. System configuration. Key: M = micellar mobile phase; R = recovery mobile phase; W = wash mobile phase; MP = analytic mobile phase.

detector with excitation at 240 nm. The emission wavelengths were selected with a 360-nm bandpass filter. The flow cell volume was 25 μl. The photomultiplier tube voltage was 650 V with a range of 0.1 μA full scale.

The tricyclic anti-depressants were determined by absorbance with a photodiode array absorbance detector. The detector wavelength was set at 239 nm unless otherwise specified.

Procedure

Plasma samples were prepared by filtration through 0.45-μm Centrex disposable centrifuge filtration units (nylon membrane) obtained from Schleicher and Schuell prior to loading the autosampler (WISP, Waters Assoc., Milford, MA, U.S.A.).

RESULTS AND DISCUSSION

Improving sensitivity and selectivity

Improvements in sensitivity in column switching procedures can be realized by simply increasing the sample size and trace enrichment on-line provided there is appropriate selectivity. The problem of selectivity was the limiting factor in our earlier work[11]. Interferences from endogenous and exogenous material limited the ultimate limit of detection (LOD) that was attainable. That work employed a 3 × 1 cm I.D. enrichment column packed with 5-μm porous reversed-phase material. While the column lifetime was in the hundreds of injections, the dispersion caused by the large frits required opening the cut window thereby allowing the admission of potential interferences to the analytical column. Narrower I.D. frits (*e.g.* 2 mm) substantially reduced column lifetime.

A more optimal solution is to employ 4.6 or 3.2 mm I.D. columns with the length tailored to the individual application (usually 15–40 mm). These small columns provide less dispersion than those with larger diameters. This results in sharper peaks. Therefore, the extraction/cleanup column and the analytical column can be kept in series for a shorter period of time. These miniature columns are inexpensive enough such that they can be discarded every hundred injections.

Exogenous interferences from reagents, primarily SDS, can also limit detectibility. Since the micellar mobile phase has relatively weak eluting power, hydrophobic contaminants are retained at the head of the analytical column. These contaminants may be subsequently eluted during mobile phase switching. The degree of interference was related to the grade of the raw material employed. Interferences were evident for both absorbance and fluorescence detection. Sequanal-grade SDS provided the lowest interferences. Since this column switching procedure employs trace enrichment, the purity of all reagents is especially critical.

Selective recovery from the extraction column

In our previous work, the drug was cut onto the analytical column with micellar mobile phase as it eluted off the tail of the exclusion front. This style of recovery, illustrated in Fig. 2, has three significant disadvantages that markedly impact selectivity: (i) endogenous serum substances are eluted onto the analytical column; (ii) the micellar mobile phase is drastically different from the analytical mobile phase result-

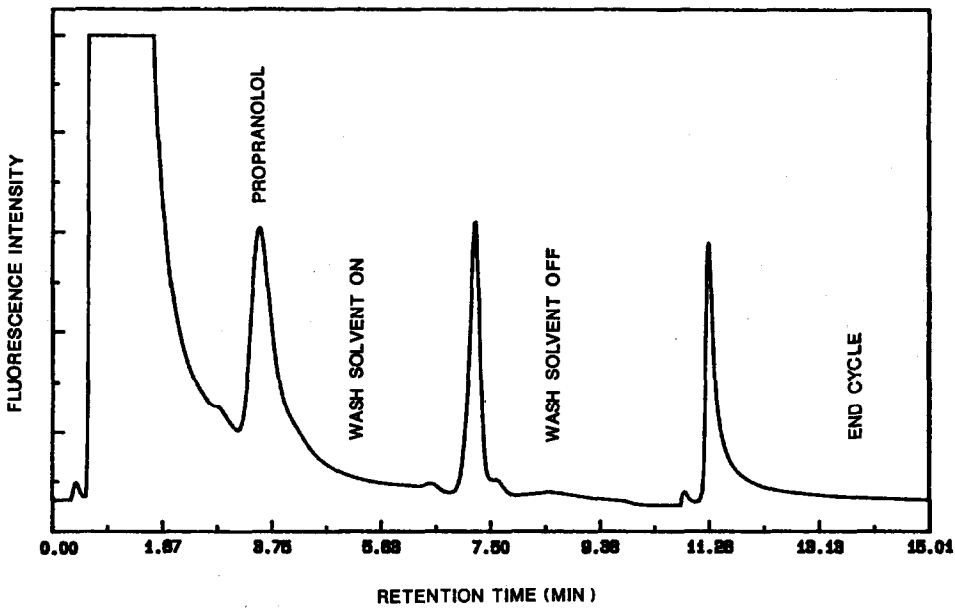

Fig. 2. Extraction column chromatogram of propranolol in blood plasma, 400 ng/ml with a common extraction and micellar recovery solvent. Column: 30 × 10 mm I.D. (cyanopropyl bonded phase on 5 μm porous silica). Injection size: 100 μl. Extraction mobile phase: acetonitrile–water (8:92, v/v) containing 40 m*M* SDS, 10 m*M* sodium dihydrogenphosphate, 10 m*M* sodium hydrogenphosphate; flow-rate: 2 ml/min. Wash mobile phase: acetonitrile–water (70:30, v/v) containing 40 m*M* SDS and 10 m*M* sodium dihydrogenphosphate; flow-rate: 2 ml/min.

ing in a large baseline shift since as much as 4 ml may be cut onto the analytical column; and (iii) the relatively weak eluting power of the micellar mobile phase results in the retention and buildup of hydrophobic substances on the analytical column.

These disadvantages were overcome by providing for a more selective elution of the extraction column. This was achieved in the following stepwise manner: (i) inject a plasma sample under micellar conditions; (ii) select the micellar solvent and column to ensure significant retention of the drug; (iii) after clearance of the proteinaceous material, elute the drug with a non-micellar recovery mobile phase; (iv) the extraction and analytical columns are placed in series and the drug recovered onto the analytical column; (v) switch to the analytical mobile phase and perform the separation on the analytical column; and (vi) simultaneously with step (v) wash and re-equilibrate the extraction column with micellar mobile phase.

Following this protocol results in the ordering of mobile phases flowing through the extraction column as follows: (i) micellar solution (extraction solution; the solvent that washes endogenous material off the column while permitting analyte retention); (ii) recovery solution (the solvent that elutes the analyte off the extraction column onto the analytical column); (iii) wash solution (a strong solvent employed to clean-up the extraction column).

A chromatogram of the extraction column run under the above conditions is shown in Fig. 3. Compared to Fig. 2, the drug is further removed from the proteinaceous front and the peak is considerably sharpened. Two hundred separate cycles were

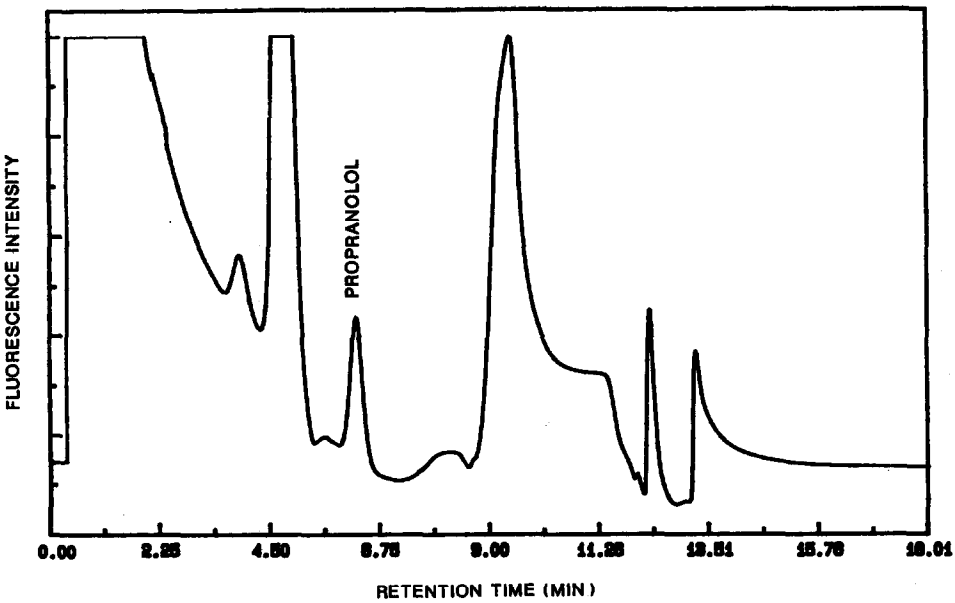

Fig. 3. Extraction column chromatogram of propranolol in blood plasma, 100 ng/ml with a selective recovery solvent. Column: 40 × 4.6 mm I.D. (phenylpropyl bonded phase on 5 μm porous silica). Injection size: 200 μl. Extraction mobile phase: aceonitrile–water (8:92, v/v) containing 60 m*M* SDS, 10 m*M* sodium dihydrogenphosphate, 10 m*M* sodium hydrogenphosphate; flow-rate: 2 ml/min. Recovery mobile phase: acetonitrile–water–acetic acid–triethylamine (42:58:0.2:0.04, v/v); flow-rate: 2 ml/min. Wash mobile phase: acetonitrile–water (70:30, v/v) containing 60 m*M* SDS and 10 m*M* sodium dihydrogenphosphate; flow-rate: 2 ml/min.

performed while monitoring the retention time of propranolol on the extraction column. No significant shifts in retention time were found. The cut window remained constant over a span of 200 injection cycles, a requirement for this form of analytical procedure.

The qualifications of the recovery solvent are straight forward. The solvent is usually an aqueous dilution of the analytical mobile phase. This measure generally provides for solvent focusing on the head of the analytical column.

The end result of all of these measures is a dramatic sharpening of the peak or peaks of interest both on the extraction as well as the analytical column. Both sensitivity and selectivity are improved. The chromatogram of propranolol, Fig. 4, is substantially improved compared to previous work[11]. The LOD, conservatively estimated at 0.5 ng/ml, is improved by a factor ten.

Routine operation of the method

The method was applied towards the determination of propranolol in blood plasma from nine subjects receiving a single oral dose of 80 mg propranolol hydrochloride. Plasma samples were drawn just prior to dose and at 0.5, 1, 2, 3, 4, 5, 6, 8, 10 and 12 h post dose. The mean observed peak drug level was 31 ng/ml. The limit of quantitation (LOQ) was set at 1.0 ng/ml where the relative standard deviation (R.S.D.) approached 10%. The complete results of the pharmacokinetic studies will be published elsewhere.

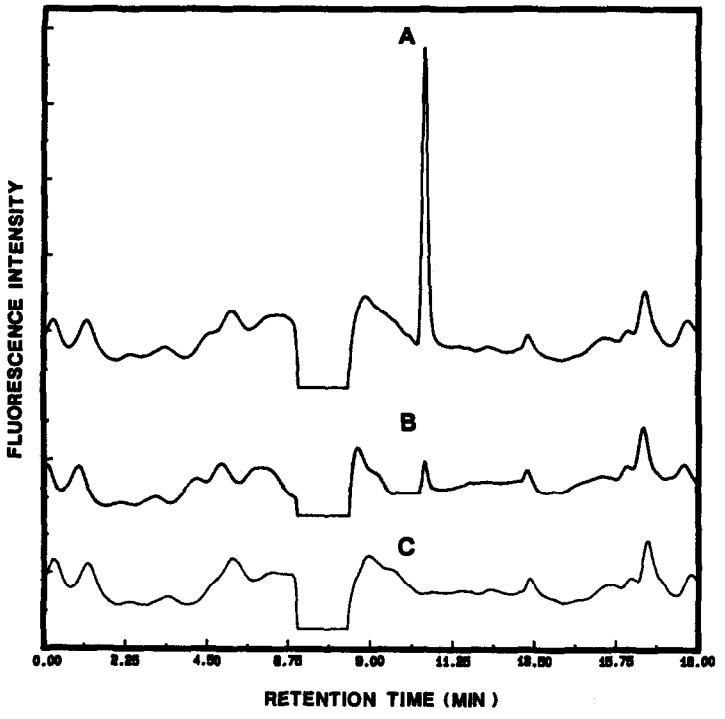

Fig. 4. Multidimensional chromatograms of propranolol. (A) 10 ng/ml; (B) 1 ng/ml; and (C) blank, all in blood plasma. Extraction column, extraction mobile phase, recovery mobile phase and wash mobile phase as in Fig. 3. Injection size: 200 μl. Analytical column: Spheri-5, RP-18, 22 cm × 4.6 mm I.D. cartridge column. Analytical mobile phase: acetonitrile–water–acetic acid–triethylamine (58:42:0.5:0.1, v/v); flow-rate: 1.5 ml/min.

A typical overnight automated run consisted of 25 plasma samples, 16 spiked plasma standards, and 4 spiked plasma quality control samples. All samples and controls were randomized and bracketed by randomized standards.

While extraction columns could be used for 200 analyses, the volume of plasma available for analysis was limited. In order to eliminate the possibility of sample loss due to column failure, the extraction column was changed every two days or 100 injections. The cost of the 40 cm × 4.6 mm I.D. extraction column was U.S.$ 0.90/injection. The same analytical column was used throughout these studies with no measurable deterioration.

The concentration of each sample was calculated from a least-squares regression plot of an eight-point standard curve. A standard curve was run in duplicate with each automated run. The precision and accuracy of the assay are shown in Table I. At propranolol concentrations above 25 ng/ml, the assay precision was found to be less than 1.7% R.S.D. At the LOQ, the R.S.D. was 9.3%.

Same day precision was assessed in two ways. Thirty percent of all samples were run in duplicate on each day. The average R.S.D. values for duplicates on a single day ranged from 0.7 to 11.7% (mean 3.8%) for nine days of experiments. On one partic-

TABLE I

STANDARD CURVE STATISTICS OF PROPRANOLOL IN PLASMA

Predicted concentrations from regression analysis.

Day	*Actual concentration (ng/ml)*							
	1.00	*2.00*	*5.00*	*10.0*	*25.0*	*50.0*	*75.0*	*100*
1	1.05	2.06	4.88	9.71	25.3	50.2	75.2	99.8
2	1.09	1.83	5.20	9.69	24.6	49.5	76.1	100
3	1.02	2.02	4.96	9.89	25.0	50.1	75.1	
10	0.85	2.23	5.40	9.48	24.5	50.2	75.4	99.9
11	1.00	2.01	5.10	9.78	25.2	50.1	72.9	102
14	1.03	2.00	4.98	9.77	24.8	50.1	75.5	
15	1.09	2.00	4.74	9.71	24.5	49.8	75.8	101
18	1.00	2.12	5.14	9.23	24.3	50.7	75.4	100
19	1.21	1.98	4.94	9.25	24.1	50.2	75.8	100
Mean (ng/ml)	1.04	2.03	5.04	9.61	24.7	50.1	75.2	100
R.S.D. (%)	9.3	5.3	3.9	2.5	1.7	0.65	1.2	0.7
Accuracy (%)	3.8	1.5	0.7	−3.9	−1.1	0.3	0.2	0.4
Same day[a] R.S.D. (%)	1.5	7.4	0.3	2.9	2.6	2.2	0.6	1.6

[a] Calculated from triplicate runs of each plasma standard during a single day.

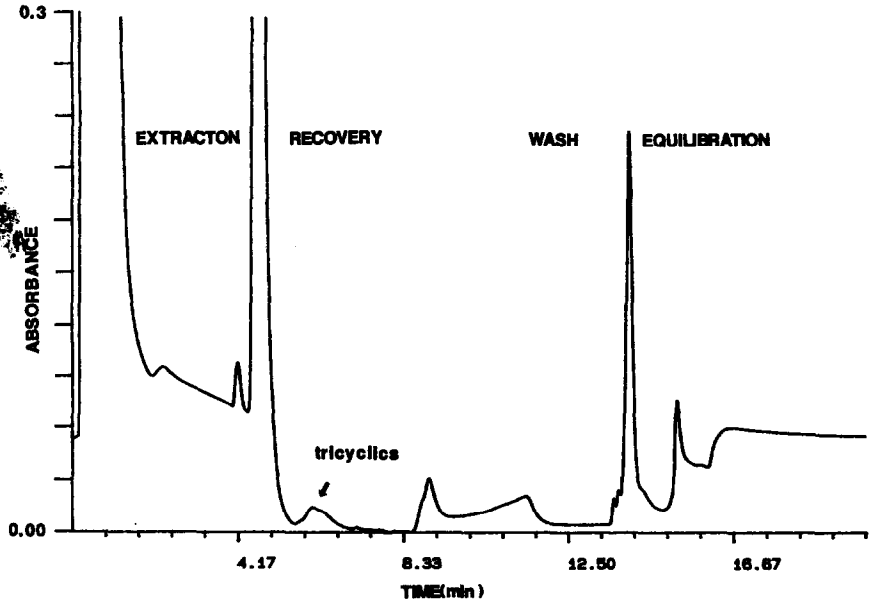

Fig. 5. Extraction column chromatogram of tricyclic anti-depressants in plasma. Extraction column: 40 × 4.6 mm I.D. polystyrene–divinylbenzene, 10 μm spherical polymer). Extraction mobile phase: acetonitrile–water–triethylamine (8:92:0.2, v/v) containing 60 m*M* SDS, 10 m*M* sodium dihydrogenphosphate and 10 m*M* sodium hydrogenphosphate; flow-rate: 2 ml/min. Recovery mobile phase: acetonitrile–methanol–water (56:12:32, v/v) containing 8 m*M* SDS, 8 m*M* sodium dihydrogenphosphate and 1 m*M* phosphoric acid; flow-rate: 2 ml/min. Wash mobile phase: acetonitrile–water (90:10, v/v) containing 60 m*M* SDS; flow-rate: 2 ml/min.

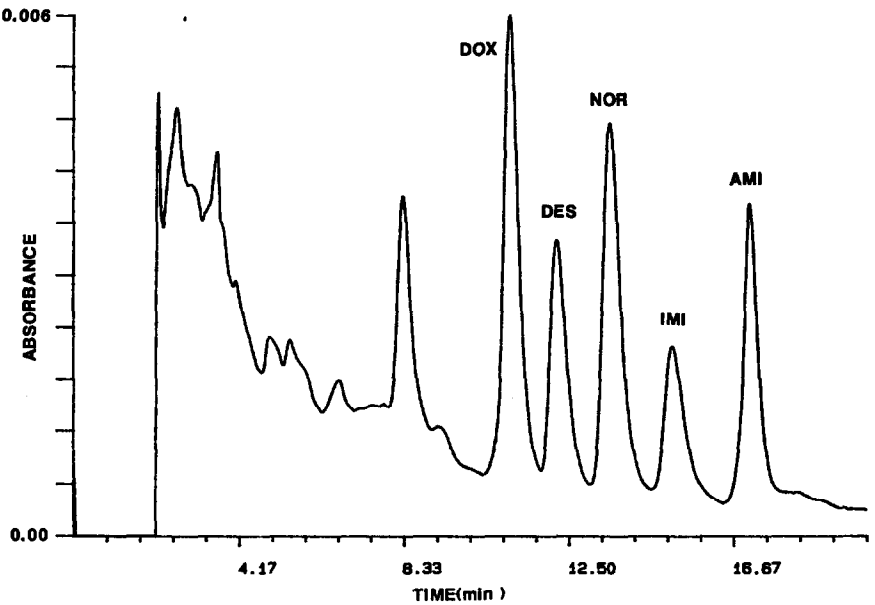

Fig. 6. Multidimensional chromatogram of tricyclic antidepressants (270 ng/ml) in blood plasma. Extraction column, extraction, recovery and wash mobile phases as in Fig. 5. Analytical column: Spheri-5, RP-18, 22 cm × 4.6 mm I.D. cartridge column. Analytical mobile phase: acetonitrile–methanol–water–triethylamine (56:12:32:0.08, v/v) containing 8 m*M* SDS, 8 m*M* sodium dihydrogenphosphate and 1 m*M* phosphoric acid. DOX = doxepin; DES = desipramine; NOR = nortriptyline; IMI = imipramine; and AMI = amitriptyline.

ular day, the entire eight-point calibration curve was run in triplicate. These data are also reported in Table I.

No carryover between sample injections was observed for the concentration range studied (0–100 ng/ml).

MULTICOMPONENT ANALYSIS

Multicomponent analysis is facile under a set of well defined conditions: (i) the compounds elute off the extraction column in a single band; (ii) the compounds are separable on the analytical column; (iii) ideally, the mechanism of separation differs in each of the columns; and (iv) detection is optimized.

For the determination of tricyclic anti-depressants, the pH of the mobile phase could be exploited along with various mobile phase additives to accomplish the first three conditions. Previous reports[13] for the solid phase extraction of these compounds from blood plasma used high pH. In that off-line sample preparation procedure, the extraction columns are discarded after only one use. For our application, multiple cycles are required so polymeric phases were used.

The selective recovery of the tricyclics from a polymeric extraction column is illustrated in Fig. 5. The five components are separated as a single peak from the solvent front of the recovery mobile phase. The recovery is accomplished by a pH

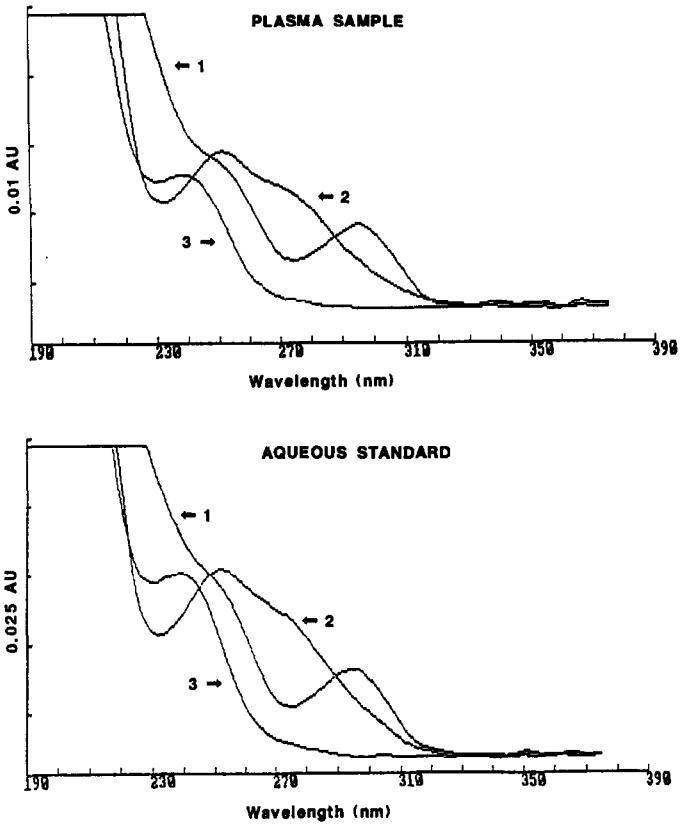

Fig. 7. UV spectra of (1) doxepin, (2) imipramine and (3) amitriptyline captured on-the-fly at the peak maxima from multidimensional chromatograms of the drugs from plasma (top) and aqueous spiked samples (bottom).

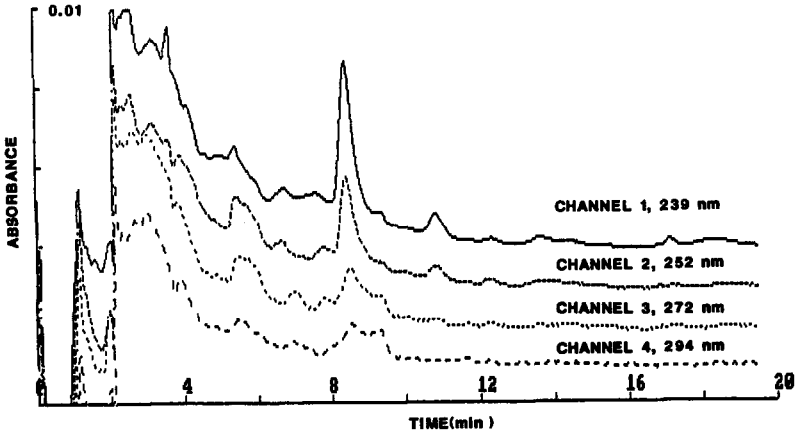

Fig. 8. Blank plasma multidimensional chromatograms obtained under conditions described in Fig. 6.

change; the extraction solvent pH was adjusted to 10 with triethylamine (TEA) while the recovery solvent was formulated without the base. It was important to ensure that none of the high-pH extraction solvent was passed onto the silica-based analytical column. No problems were found here. The selection of the analytical mobile phase was straight forward. TEA was added to minimize peak tailing and facilitate elution at pH 6.0.

A column-switched separation of an injection of 500 μl of blood plasma containing 270 ng/ml of each tricyclic anti-depressant is shown in Fig. 6. Monitored at a compromised wavelength of 239 nm, doxepin and desipramine showed positive interferences of 18 and 10%, respectively. The other drugs gave recoveries of 100%. The UV spectra of the tricyclics and their metabolites (Fig. 7) indicate opportunities for monitoring at wavelengths other than 239 nm. Indeed, using 294 nm for doxepin gives a 100% recovery. The same holds true for desipramine monitored at 272 nm. Examination of the blank plasma runs (Fig. 8) at four selected wavelengths clearly indicates these interferences at 8.5 and 10.3 min. That information, coupled with the spectroscopic data, provides a ready and simple solution. With diode array technology, timed programmed-wavelength switching is simple and gives no baseline shifts. It becomes simple to optimize the wavelength for selective and sensitive results. Opportunities to further reduce matrix interferences through chemometric techniques like spectral suppression[14] present challenges for future studies.

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