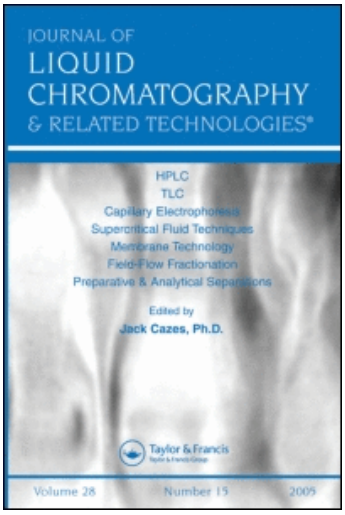

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Khanh H. Bui[a]; Scott B. French[a]
[a] ICI Pharmaceuticals Group ICI Americas, Inc., Wilmington, Delaware

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DIRECT SERUM INJECTION AND ANALYSIS OF DRUGS WITH AQUEOUS MOBIL PHASES CONTAINING TRIETHYLAMMONIUM ACETATE

KHANH H. BUI AND SCOTT B. FRENCH
ICI Pharmaceuticals Group
ICI Americas, Inc.
Wilmington, Delaware 19897

ABSTRACT

The use of aqueous mobile phases containing only moderate concentrations of triethylammonium acetate (TEAA) permits the direct injection of untreated plasma sample onto the chromatographic column without causing the precipitation of proteins and the consequential clogging of the analytical column. Furthermore, the TEAA modifier can be used in place of the organic modifier to manipulate the retention of the drugs. The potential usefulness of this technique for the analysis of drugs in biological fluids is demonstrated and discussed.

INTRODUCTION

Chromatographic analysis of drugs in biological fluids such as serum or plasma generally requires precolumn sample preparation to separate the analytes from the protein matrix. The removal of proteins is necessary because proteins denature and precipitate when injected into conventional mobile phases containing organic modifiers such as acetonitrile and methanol and clog the injector port and/or the analytical column.

Commonly used sample pretreatment steps usually involve time consuming and labour intensive protein precipitation and/or liquid or solid phase extraction procedures which not only lengthen the assay procedure and introduce additional sources of error but may also result in low and irreproducible recoveries of the analytes. Consequently, there is tremendous impetus either to automate or to circumvent altogether the above steps. Recent efforts to automate the above steps involve either the use of robotics, which requires considerable set up time and expensive instrumentation (1,2), or the use of on-line column switching techniques which although requires less expensive instrumentation, have inherent limitations such as band broadening due to the use of pre-columns containing large particles (to avoid rapid clogging of the pre-column), periodic replacement of the pre-column due to the accumulation of proteinaceous materials and long analysis times due to the additional equilibration, purge, and wash steps (3,4).

Recently, two different chromatographic approaches have been developed which permit the direct injection of untreated biological fluids onto the analytical column. The first approach pioneered by Pinkerton et al. (5,6) involved the development of novel stationary phases which allow reversed-phase separation of drugs present in the serum without prior sample cleanup. These novel stationary phases which are termed internal surface reversed-phase silica supports have a unique hydrophobic internal suface and a unique hydrophilic external surface, which permit proteins to be excluded while allowing the smaller size drugs to be retained and separated.These stationary phases, besides being very expensive, do require the use of organic modifiers which when used at high enough composition (to elute

highly nonpolar compounds) still result in the denaturation and precipitation of proteins.

Another approach involves the use of non-ionic and ionic micellar solutions as the mobile phases (7-10). These micellar solutions, because of their unusually powerful solubilizing properties, allow the direct injection of untreated serum and urine onto the chromatographic column without the precipitation of proteins and the consequential clogging of the analytical column. One major limitation of this approach was the moderate chromatographic performance caused by the analytes' restricted mass transfer processes (15).

Aqueous mobile phases containing only triethylammonium acetate modifier permits the direct injection of untreated plasma sample onto the chromatographic column without causing the proteins to precipitate. Furthermore, triethylammonium acetate modifier can be used in place of organic modifier to manipulate the retention of the drugs. The potential usefulness of this technique for the analysis of drugs via direct injection of biological fluids onto the chromatographic column as well as the mechanism for their retention on silica gel stationary phases is demonstrated and discussed.

EXPERIMENTAL

Reagents: ICI 118551, ICI 182359, ICI 160181, Atenolol and Propranolol were obtained from Stuart Pharmaceuticals. Metoprolol was obtained from Ciba Geigy Inc. . Hexanophenone and triethylamine (HPLC grade) were obtained from Pierce Chemical. Glacial acetic acid and water (HPLC grade) were obtained from J.T. Baker.

Apparatus:The HPLC system consisted of two high pressure pumps (Shimadzu LC-6A), a system controller (Shimadzu SIL-6A), an automatic sample injector (Perkin-Elmer ISS-100), and either a UV variable wavelength detector (Shimadzu SPD-6A) or a flourometric detector (Shimadzu RF-530). All chromatograms were recorded with a Linear model 1200 recorder and an HP 3350 data aquisition system. The HPLC analytical column was either a 5 um Partisphere silica cartridge column (4.6 mm x 12.5 cm) (Whatman, Clifton, NJ) or a 3 um Pecosphere silica cartridge column (4.6 mm x 8.3 cm) (Perkin-Elmer,Norwalk,CT).

Procedure: The 1% (v:v) triethylammonium acetate buffer solution was prepared by mixing 10 ml of triethylamine with HPLC grade water , titrating to a pH of 4 with glacial acetic acid, transferring the solution to a one liter volumetric flask and diluting to the mark with HPLC grade water.Triethylammonium acetate buffer solutions of lesser concentrations were prepared by appropriate dilution of the above solution. Plasma was obtained by centrifuging blood obtained from in-house dogs and rats. The plasma was filtered through a 0.45 um membrane filter prior to use. Stock solutions of each drug were prepared in HPLC grade methanol. Plasma matrix standards were prepared by spiking appropriate quantity of the stock solutions to blank plasma.

RESULTS AND DISCUSSION

Direct Injection of Plasma Sample

The chromatographic efficiency as well as the column back pressure monitored during 100 injections of untreated plasma spiked with propranolol are illustrated in Figure 1. It is

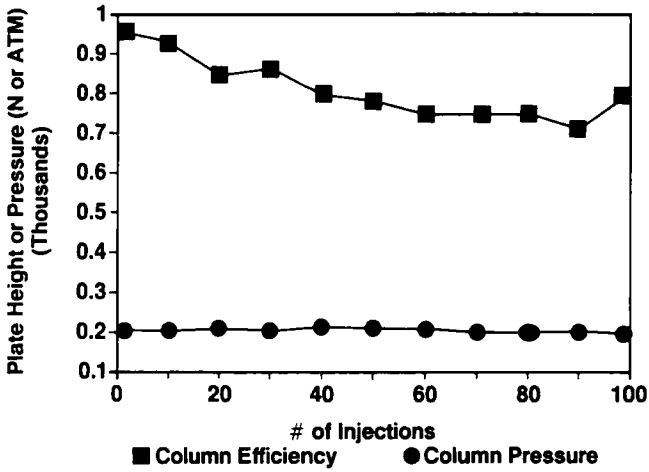

Figure 1. Plots of number of injections versus column efficiency and pressure. Chromatographic conditions: column, 5 μm Whatman Partisphere silica (4.6 mm by 125 mm); mobile phase, 0.15% TEAA buffer (pH = 4); flow rate, 2 ml/min.; UV wavelength, 240 nm; injection volume, 20 μl of 1 μg/ml spiked plasma.

apparent that no increase in column back pressure as well as no significant decrease in column efficiency was observed following these injections. This column was subsequently used throughout the study - receiving more than 200 additional injections of untreated plasma - without any noticeable change in column efficiency and/or back pressure.

The chromatographic separations of several beta-blockers as well as some analgesic agents in dog plasma are illustrated in Figure 2 and Figure 3 respectively. Since the analgesic agent naproxen is acidic and the beta-blockers are basic, it is apparent that this technique is applicable to both cationic as well as anionic drugs. Furthermore Figures 2 and 3 illustrate

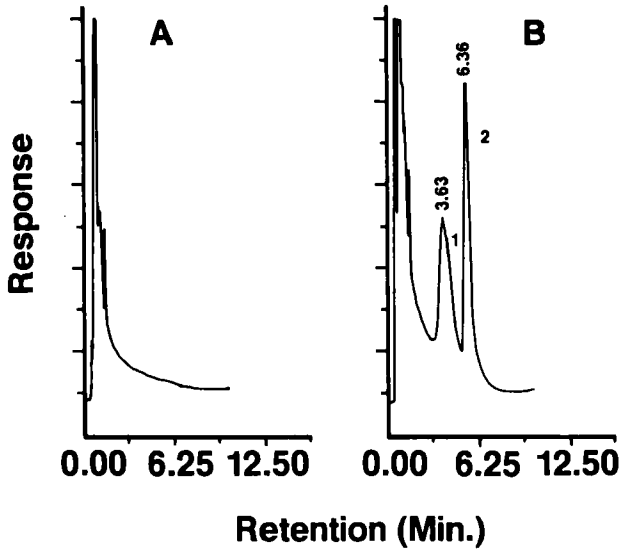

Figure 2. Chromatograms of (A) blank dog plasma, (B) dog plasma spiked with (1) 10 μg/ml antipyrine and (2) 10 μg/ml naproxen. Chromatographic conditions: column, 5 μm Whatman Partisphere silica (4.6 mm by 12.5 cm); mobile phase, 0.10% TEAA buffer (pH = 4); flow rate, 2 ml/min.; UV wavelength, 240 nm; injection volume, 10 μl.

that these drugs, which were completely eluted within 10 minutes were well resolved from the proteinaceous plasma interferences. Indeed, the blank plasma samples in Figures 2 and 3, which were detected by two different types of detectors (UV and Fluorometric) produced background responses which returned to baseline after 4 minutes. Consequently, since no increase in column backpressure and no deterioration of column efficiency as well as no long eluting peaks were observed following repeated injections of plasma samples, it can be assumed that the proteinaceous components of the plasma eluted very early on the

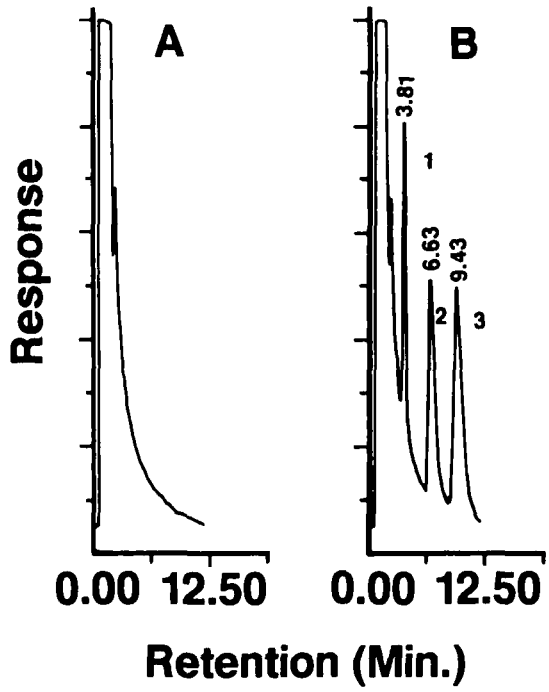

Figure 3. Chromatograms of (A) blank dog Plasma, (B) dog plasma spiked with (1) 10 μg/ml atenolol, (2) 10 μg/ml propranolol, and (3) 10 μg/ml metoprolol. Chromatographic conditions: column, 3 μm Perkin Elmer Pecosphere silica (4.6 mm by 8.3 cm); mobile phase, 0.01 % TEAA buffer (pH = 4); flow rate, 2ml/min; fuorescent excitation wavelength, 270 nm; emmission wavelength, 310 nm; injection volume, 20 μl.

silica gel stationary phase with the aqueous ion-pair mobile phases used.

A typical calibration curve for the determination of propranolol in rat plasma using this direct injection approach is illustrated in figure 4. The curve exhibits satisfactory linearity in the range covered with a correlation coefficient value of 0.996.

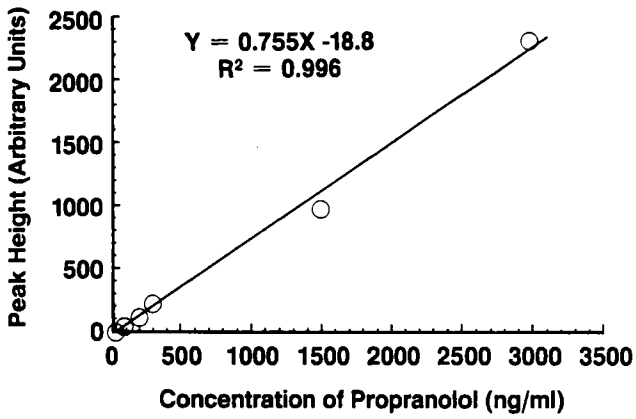

Figure 4. Calibration curve of propranolol in rat plasma. Chromatographic conditions are similar to those in figure 3.

The unusual selectivity of this technique is illustrated in figure 5 which shows the chromatographic separation of ICI 118551, a selective β - adrenoreceptor antagonist being developed for human use in non-cardiovascular indications, from its known circulating metabolites and plasma interferences. Figure 5 illustrates not only the separation of structurally similar compounds such as a drug and its metabolites, but also the separation of a pair of diastereomers as well. Indeed peaks B and C in the figure represent the chromatographic peaks of the diastereomers of ICI 160181 (see Figure 5 for the structure of the diastereomers).

Mechanism of the separation and effects of additive concentration

The effect of the triethylammonium acetate concentration (expressed as volume %) on the retention profiles of basic neutral and acidic test solutes are illustrated in Figure 6. For

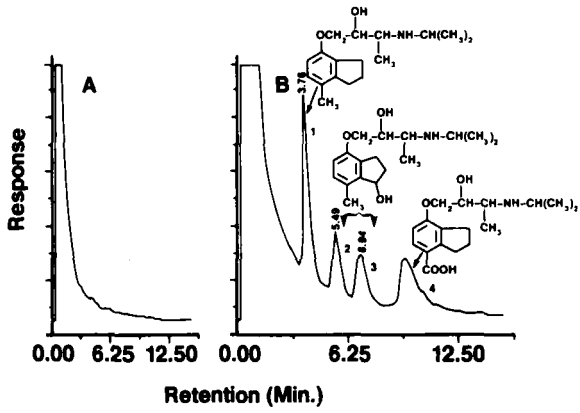

Figure 5. Chromatograms of (A) blank dog plasma, (B) dog plasma spiked with (1) 5 μg/ml ICI 118551, (2 & 3) 5 μg/ml ICI 160181 diastereomers, and (4) 5 μg/ml ICI 182359. Chromatographic conditions, 3 μm Perkin Elmer Pecosphere silica (4.6 mm by 8.3 cm); mobile phase, 0.01% TEAA buffer (pH = 4); flow rate, 2 ml/min.; wavelength, 240 nm; injection volume, 20 μl.

the basic and neutral solutes, the capacity factor decreases with increasing triethylammonium acetate concentration, while for the acidic solute, the capacity factor increases to a maximum value and then decreases with increasing triethylammonium acetate concentration. This difference in the retention profile of the acidic solute and those of the basic and neutral solutes can be easily explained by the solutes mode of interactions with the silca support. It is known that the silica gel surface contains siloxane bridges and several types of silanol groups which are capable of manifesting different types of interactions (14). For instance, the retention on a silica support can be caused by hydrophobic interactions with siloxane bridges (particularly

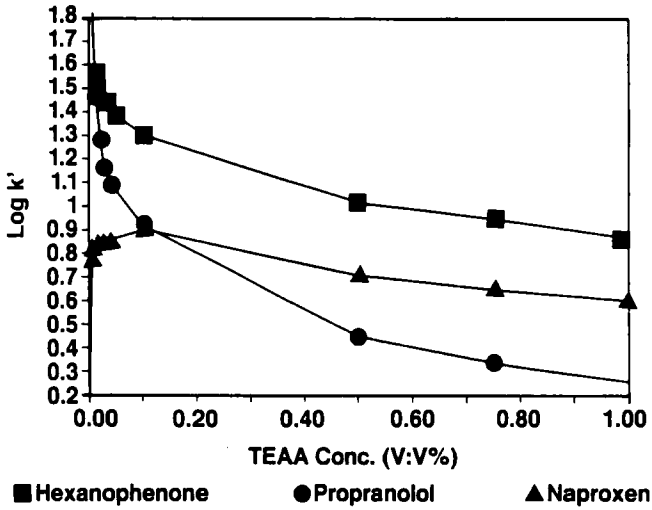

Figure 6. Plots of log K' versus buffer concentration of (□) hexanophenone, (+) propranolol, and (◇) naproxen. Chromatographic conditions: column, 5 um Whatman Partisphere silica (4.6 mm by 125 mm); wavelength, 240 nm; flow rate, 2 ml/min; injection volume, 10 ul; analytes concentration, 10 ug/ml.

important when aqueous mobile phases are used), ion exchange interactions with intermolecular hydrogen bonding and highly acidic silanol groups, and other silanophilic interactions (such as hydrogen bonding) with the various silanol groups (11-13). The observed retention profile of the acidic solute, naproxen is similar to that observed in reversed phase LC when ion interaction agents are added. In fact a somewhat analogous mechanism may be operative in this experiment. In a pure water mobile phase, the acidic solute was repelled by the acidic sites on the silica suface and eluted with the solvent front. Upon addition of triethylammonium acetate, the retention of naproxen

was achieved due either to its formation of a neutral paired ion complex or the dynamic coating of the acidic silanol sites by triethylamine. With further addition of triethylammonium acetate, the retention of naproxen decreases due to the competition of triethylammonium and/or acetate ions for adsorption sites - hydrophobic or silanophilic - on the silica surface.

Likewise, the decrease in the retention of the neutral and basic solutes with increasing concentration of triethylammonium acetate was probably caused by the competition for the various adsorption sites on the silica surface by triethylammonium acetate. Consequently, the variation of the mobile phase additive concentration, which can profoundly affect the retention of the acidic, neutral and basic solutes, can be used in place of the organic modifier to control the selectivity as well as the analysis time of the separation. Furthermore, since numerous types of interactions can be caused by the silica surface, unusual selectivities can be achieved with this technique as illustrated by the separation shown in Figure 5.

Advantages and Limitations

Compared to the two previous approaches for direct injection of biological samples, this approach offers the advantage of using both inexpensive and common mobile and stationary phases. Furthermore no organic modifier is used in this approach to either affect the retention as in the case of the Pinkerton's approach (5,6) or to improve the efficiency as in the case of the micellar approach (8-10). The use of organic modifiers, at high enough concentration, would undoubtedly promote the denaturation and precipitation of proteins and would lead to the consequential clogging of the column. Other problems associated with the use

of aqueous/organic mobile phases such as the generation of air bubbles caused by the instrumental mixing of organic modifier and water during the analysis and the high column back pressure caused by the inherent high viscosity of the organic/aqueous mobile phases are removed. The problem due to the high compressibility of organic modifiers which amplify the pump noises and which is very deleterious to pressure sensitive detectors such as the refractive index and electrochemical detectors is also eliminated.

Compared to conventional HPLC biological fluid asssay, possible disadvantages of this approach include: (i) preconcentration of the analytes to increase their detectability can't be performed since there is no sample preparation step involved, (ii) possible lower detector sensitivty due to the lower UV transparency caused by the interfering ion-pair reagents, and (iii) shorter column lifetimes due to the injection of unusually dirty samples.

This approach is being applied to various classes of drugs to expand its usefulness. Furthermore, evaluation of alternate stationary phases such as bonded silica packings (i.e., cyano and diol), alumina packings as well as other types of ion pair reagents to expand the versatility as well as the potential of the technique is currently being investigated.

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