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Switching-valve–filter technique for the direct injection and analysis of drugs in plasma using high-performance liquid chromatography☆

E.B. Asafu-Adjaye*, Syang Y. Su☆☆, Gerald K. Shiu

Generic Drugs Research, OGD, CDER, FDA, 200 C Street S.W., Washington, DC 20204, USA

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Abstract

An automated technique involving switching valves and a filter assembly has been developed and evaluated for the on-line precipitation of proteins and peptides from plasma samples. In the set-up, the proteins were precipitated on-line by injecting the plasma sample into a stream of organic precipitating agent. The precipitates so formed are filtered on-line by a set of filter assemblies consisting of ordinary in-line HPLC solvent filters. Evaluation of the technique was performed using ibuprofen and a mixture of three estrogens, estradiol, equilin and estrone, spiked in dog plasma. The coefficients of variation (C.V.) for system suitability parameters were below 10%. Absolute recovery of ibuprofen in plasma ranged from 80% for 100 μg/ml to 114% for 5 μg/ml spiked concentrations, respectively. Resolution for equilin and estrone, two closely eluting peaks, was 1.79 (C.V. = 5.8%, $n = 7$). The switching-valve–filter assembly had no significant effect on the efficiency of the HPLC system.

1. Introduction

High-performance liquid chromatographic analysis of drugs in biological matrices such as serum or plasma, urine, and tissues, usually involves treatment of the sample before injection into the chromatograph. Pre-treatments of sample are performed to remove particles that may clog the column along with endogenous compounds that will interfere with the analysis. Typical treatments include precipitation, liquid–liquid extraction and solid-phase extraction. Such conventional procedures may take one to several hours and may involve tedious and complex steps such as vortex-mixing, centrifugation, drying and sample reconstitution. Because of the many steps and extensive sample handling, contamination and sample loss are not unusual.

One approach to solving these problems is direct sample injection. This approach can also be automated, making it attractive for routine use in analytical and clinical laboratories. For the direct injection of biological samples to work, it is important that endogenous substances, especially proteins and peptides, do not foul the HPLC column. Most direct sample injection methods get around this problem by making use

* Corresponding author.

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☆☆ Present address: Baker Norton, 50 N.W. 176th St., North Miami Beach, FL 33150, USA.

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of mobile phases containing reagents such as micelles, or surfactants that are able to maintain proteins in solution [1–5], employing pre-columns to remove the interferents, or to hold and concentrate the analyte while the contaminants are allowed to go on to waste, before the analyte reaches the analytical column. The latter methods usually make use of some form of column switching techniques [6–9]. Of late, a third means of direct sample injection has been introduced. This involves the use of special columns such as Regis' semi-permeable surface, Supelco's Hisep® and Pinkerton's internal-surface reversed phase [10,11].

In this paper, we describe a new automated direct sample injection approach that uses an assembly of switching valves and in-line filters to clean up plasma samples prior to injection into the chromatograph. The assembly is in line with the analytical column, and prior to analysis the sample is injected into a stream of organic solvent to precipitate proteins and peptides. The precipitates are removed from the stream by in-line filters and then switched onto the analytical column without interrupting the operation of the HPLC. The system was evaluated using dog plasma spiked with ibuprofen or estrogen mixtures.

2. Experimental

2.1. Instrumentation

The liquid chromatograph consisted of Spectra-Physics (Santa Clara, CA, USA) components: Model SP8880 autosampler, SP8800 ternary solvent delivery system, SP8500 dynamic mixer and SpectraFocus UV-Vis forward scanning detector. The mobile phase was degassed on-line with a Shodex (Tokyo, Japan) DEGAS KT-35M degassing device. The necessary valves and column switching were provided by Waters Automated Valve System (WAVS) (Millipore, Milford, MA, USA). The WAVS also provided a means of switching, on and off, an auxiliary pump plugged into its auxiliary AC outlet. The auxiliary pump was a Beckman (Houston, TX, USA) 110B solvent delivery module. The operation of the whole system was under the control of an IBM PS/2 model 70 386 computer running Spectra-Physics' Autolab® software. Data processing was performed with Spectra-Physics SpectraStation® software. The analytical column was a 250 × 4.6 mm I.D., 5 μm Beckman Ultrasphere C_{18} operated at ambient temperature. This was coupled to a Brownlee 2 cm C_{18} cartridge guard column purchased from Applied Biosystems, Inc. (San Jose, CA, USA) The in-line solvent filter holders and their filter elements were purchased from Upchurch Scientific (Oak Harbor, WA, USA).

2.2. Reagents and materials

Ibuprofen and the estrogen standards were obtained from USP (USP Convention, Rockville, MD, USA) and were used as received. Ibuprofen stock solutions were prepared in pH 8 0.1 *M* tris buffer and working standards prepared by dilution of the stock solution with buffer or mobile phase. Stock estrogen mixtures were made in ethanol and further dilutions were made with 95% ethanol in water or mobile phase. Plasma samples were prepared by spiking dog plasma with an appropriate aliquot of the stock solution. HPLC-grade acetonitrile was purchased from Aldrich (Milwaukee, WI, USA), triethylamine (TEA) and sodium dodecyl sulfate (SDS) from Sigma (St. Louis, MO, USA), and absolute ethanol from Midwest Grain Products (Weston, MO, USA).

2.3. System operation

The configuration of the switching-valve–filter assembly depicting the four operational modes is illustrated in Figs. 1a–d. This configuration was the one that proved to be usable after trying several others. The valves 1, 2, and 4 refer to the valves of the WAVS; 1 and 2 are six-port high-pressure pneumatic valves, while 4 is three-port low-pressure electrically switched valve. The connection of the chromatograph components to the valve–filter system is shown in Fig. 1. In operation the analytical pump delivers mobile

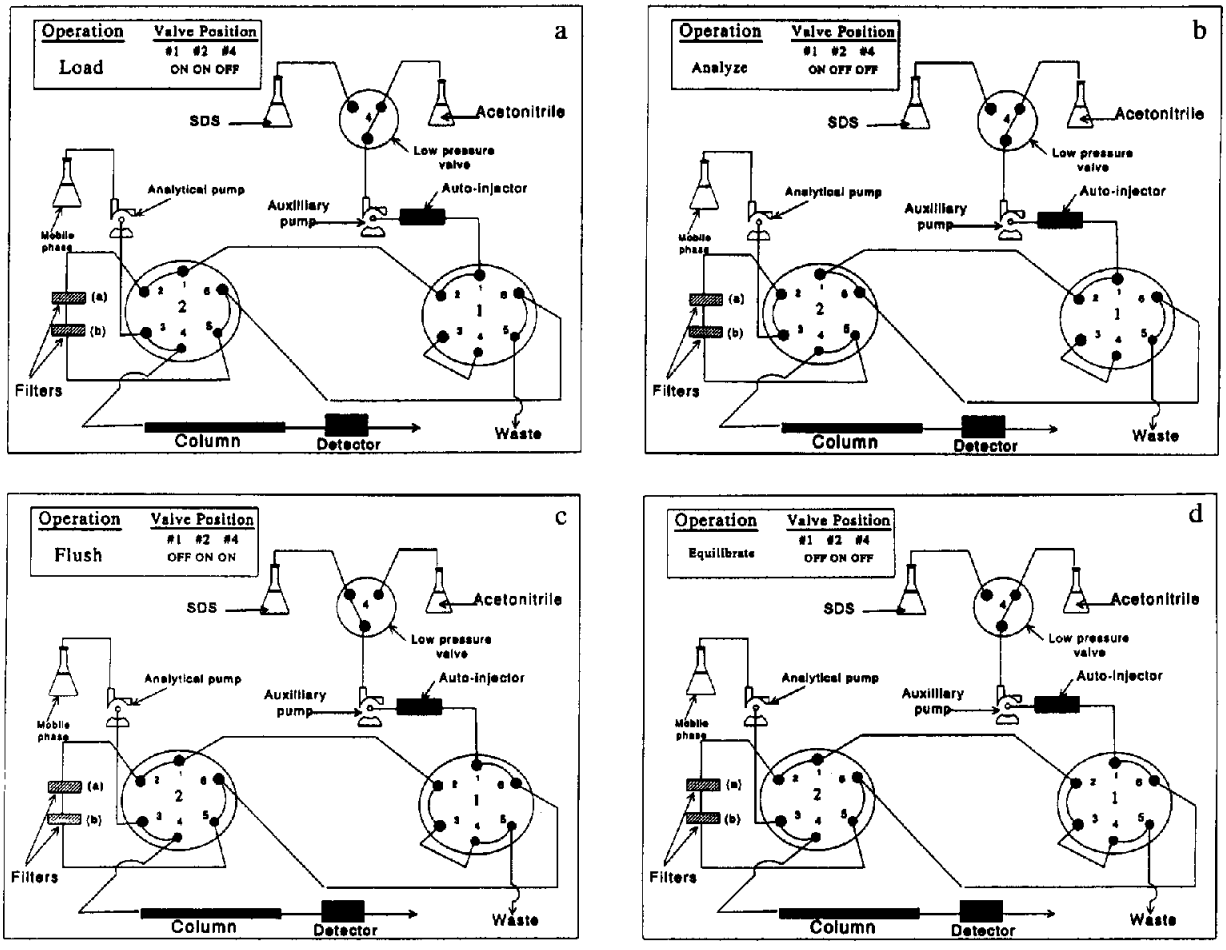

Fig. 1. Schematic diagrams of the switching valve-filter system showing set-up and flow pattern during (a) sample loading, (b) analysis, (c) filter clean-up and (d) the equilibration stages.

phase to the column continuously at a flow-rate of 1 ml/min as in any typical HPLC system. After system equilibration, sample is injected by the autosampler into a stream of acetonitrile (precipitating reagent) pumped by the auxiliary pump at a flow-rate of 0.2 ml/min. This low flow-rate ensures that there is sufficient time for precipitation of proteins to occur as the stream flows. Meanwhile valves 1 and 2 have been switched and maintained in a position, (load mode, Fig. 1a), to allow the sample stream to go through the two in-line solvent filters a and b containing 0.5-μm and 0.2-μm filter elements respectively. These two filters remove the precipitates from the stream; the clean stream is then directed to the column by the switching of valve 2 (analyze mode, Fig. 1b). The timing of this switching is critical; it must be such that the sample plug will be directed to the column just a few seconds before it leaves port 5 and goes to waste. This last minute switch ensures that only a small amount of 100% acetonitrile goes through the analytical column, preventing a large solvent front that may interfere with the separation. This behavior was the major problem in the other configurations we investigated, and that was why we rejected those configurations. A few minutes after the start of the analysis, valves 4 and 1 are switched to on and off position respectively (flush mode, Fig. 1c). Valve 2 is also

switched back to on, isolating the two in-line filters from the column. The auxiliary pump now pumps the flushing solvent, 0.1 *M* sodium dodecyl sulfate (SDS), at a higher flow-rate (2 ml/min) in a direction opposite to that of the acetonitrile. This flushes the precipitated material deposited on the in-line filters to waste thus making them reusable for further filtration of the sample. SDS was chosen as the flushing solution because it is a good solubilizing agent for proteins above its critical micelle concentration. We found that it was not necessary to flush the filters each time after injection; we could use them for *ca.* five injections before flushing becomes necessary as a result of increased pressure. Approximately five minutes before the next injection, valves 4 and 1 are switched back to the load mode and acetonitrile is pumped through the filters to replace and equilibrate them. This is the equilibrate mode (Fig. 1d).

3. Results and discussion

Ibuprofen and a mixture of estrogens were used as model compounds to evaluate the system. The estrogen mixture contained estradiol, equilin and estrone. These drugs have been extensively studied in our laboratory so the methods for their analysis were familiar. The first step was to evaluate the chromatographic system by running analyses without the switching-valve–filter system. This was done with sample solutions of the drugs in the mobile phase. Figs. 2a and b show the chromatograms for the ibuprofen and estrogen mixture respectively. Several chromatographic parameters were determined to evaluate the suitability of the system. Table 1 shows the results obtained. The results were within acceptable limits. The coefficients of variation ranged from 0.38% to 13.6% ($n = 6$). Figs. 3a and b are representative chromatograms obtained for the same samples with the switching-valve–filter assembly in place; *i.e.* sample injections were made as described in the Experimental section. Table 2 also shows the peak parameters obtained under these conditions. Except for the slight increases in the retention times, many parameters compared favorably with the results obtained without the assembly. The increases in the retention times were due to the initial low injection flow-rate. Also there were slight differences in the repeatability of some parameters as indicated by slight increases in the coefficients of variation. But on the whole, the precision was acceptable indicating that the presence and operation of the switching-valve–

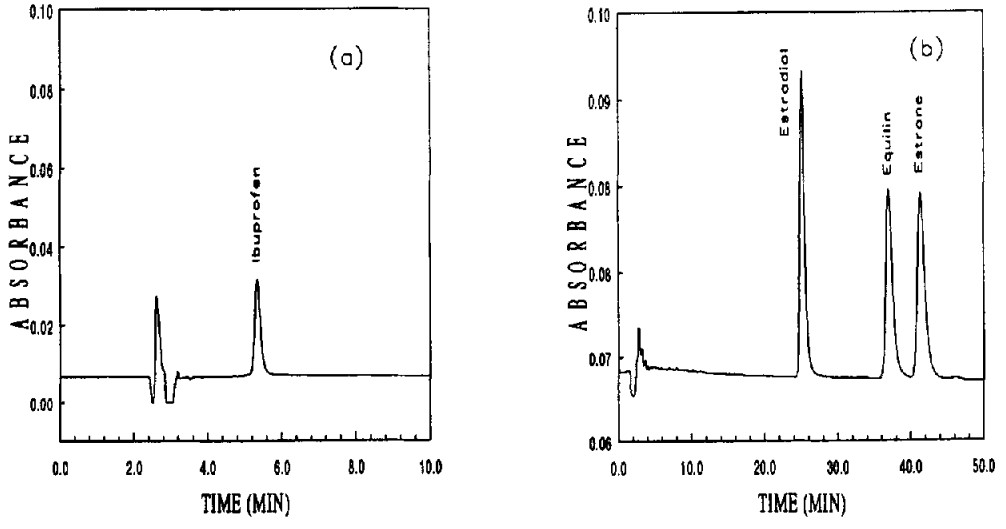

Fig. 2. Chromatograms of drug standards in mobile phase obtained without the switching valve-filter assembly; (a) ibuprofen; (b) estrogens. Conditions the same as in Table 1.

Table 1
Comparison of peak parameters of drugs in mobile phase obtained without switching-valve-filter system

Compound	Parameter	Average ($n = 6$)	S.D.	C.V. (%)
Ibuprofen	Retention time (min)	5.31	0.04	0.06
	Peak area	852000	5140	0.60
	Peak height	32700	790	2.42
	Peak symmetry	1.43	0.04	2.94
	N	5930	529	8.92
Estradiol	Retention time (min)	25.4	0.13	0.51
	Peak area	376000	10100	2.68
	Peak height	4390	87.6	1.99
	Peak symmetry	1.03	0.03	2.98
	N	7200	656	9.11
Equilin	Retention time (min)	36.5	0.34	0.94
	Peak area	331000	7580	2.29
	Peak height	2750	62.0	2.26
	Peak symmetry	1.05	0.03	3.19
	N	6650	568	8.54
Estrone	Retention time (min)	42.6	0.26	0.61
	Peak area	362000	8480	2.34
	Peak height	2710	57.3	2.11
	Peak symmetry	1.05	0.03	3.22
	N	6680	520	7.78

Conditions: Column, Beckman ultrasphere C_{18}, 250 × 4.6 I.D. mm, 5 μm particle size; flow-rate, 0.2 ml/min injection, 1 ml/min analytical; injection volume, 10 μl; ibuprofen: 26 μg/ml; mobile phase, 70% acetonitrile–30% (0.094% triethylamine in water, pH adjusted to 3 with glacial acetic acid); detection, UV 220 nm; estrogens: 10 μg/ml each of estrone, estradiol and equilin; mobile phase, acetonitrile–water (33:67, v/v); detection, UV 280 nm.

filter system would not affect the analyses. Thus, the system was further evaluated with dog plasma spiked with the model compounds. Figs. 4a and b show representative chromatograms. Comparing these chromatograms with those in Figs. 2 and 3, no significant differences between the two sets could be observed. Peak parameters measured are given in Table 3. Again, we observed no significant differences in these parameters compared with those obtained for the drugs dissolved in mobile phase, either with or without the use of the valve–filter system. Except for the number of theoretical plates, N, the coefficient of variation was below 10% for all the peak parameters, even for the peak symmetries which we had anticipated to become less for plasma samples.

The standard calibration curve for plasma spiked with 1.5 to 100 μg/ml of ibuprofen was linear as indicated by a log–log plot with a slope of 0.947 and a correlation coefficient of 0.994. The absolute recovery for ibuprofen in dog plasma ranged from 80% for 100 μg/ml (C.V. = 4.21%, $n = 6$) to 114% for 5 μg/ml (C.V. = 11.06%, $n = 6$). The effect of the system on the resolution of closely eluting components in mixtures was demonstrated by the separation of equilin and estradiol (peaks 2 and 3 in Fig. 3b). The two components were effectively resolved with an average $R_s = 1.79$ and a C.V. of 5.8% ($n = 7$).

Throughout the work, including the preliminary studies to determine the best configuration, only one column was used. Although the exact number of injections made was not determined, we believe to have made several hundreds without a significant detrimental effect on the column. The only parts of the set-up that had to be changed regularly were the set of in-let filters, and occasionally the guard column.

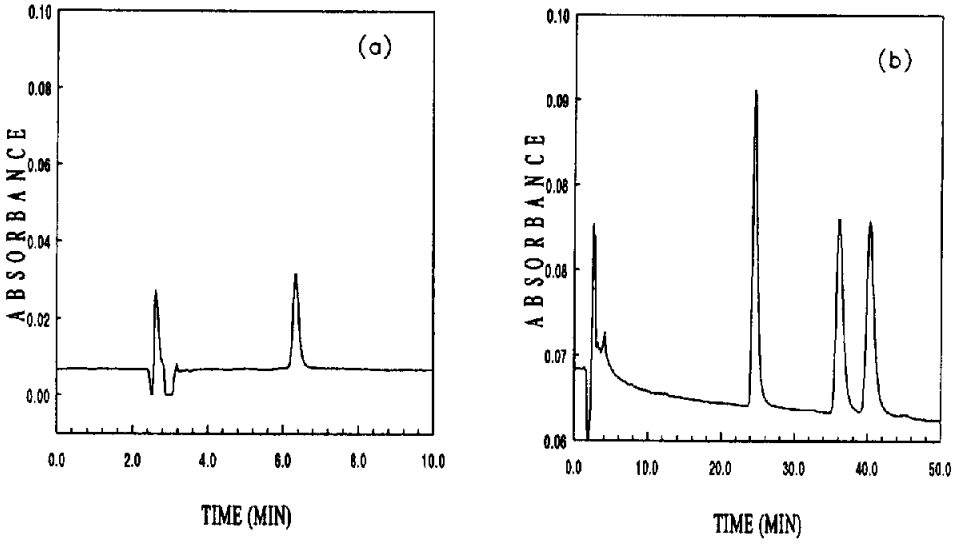

Fig. 3. Chromatograms of drug standards in mobile phase obtained with the switching valve-filter assembly; (a) ibuprofen; (b) estrogens. Conditions the same as in Table 2.

Table 2
Comparison of peak parameters of drugs in mobile phase obtained with switching-valve-filter system

Compound	Parameter	Average ($n = 6$)	S.D.	C.V. (%)
Ibuprofen	Retention time (min)	6.33	0.004	0.06
	Peak area	857000	41600	4.86
	Peak height	37900	2150	5.67
	Peak symmetry	1.04	0.03	2.53
	N	6180	733	11.9
Estradiol	Retention time (min)	25.4	0.15	0.59
	Peak area	312000	17000	5.43
	Peak height	2540	76.0	3.00
	Peak symmetry	1.13	0.04	3.81
	N	5310	446	8.41
Equilin	Retention time (min)	37.3	0.63	1.68
	Peak area	285000	12900	4.52
	Peak height	1730	84.2	4.86
	Peak symmetry	1.16	0.05	4.01
	N	4730	455	9.62
Estrone	Retention time (min)	42.6	0.28	0.66
	Peak area	298000	18400	6.17
	Peak height	1690	99.3	5.87
	Peak symmetry	1.12	0.05	4.27
	N	4710	454	9.64

Conditions: Column, Beckman ultrasphere C_{18}, 250 × 4.6 mm I.D., 5 μm particle size; flow-rate, 0.2 ml/min injection, 1 mL/min analytical; injection volume, 10 μl; ibuprofen: 26 μg/ml; mobile phase, 70% acetonitrile–30% (0.094% triethylamine in water, pH adjusted to 3 with glacial acetic acid); detection, UV 220 nm; estrogens: 10 μg/ml each of estrone, estradiol and equilin; mobile phase, acetonitrile–water (33:67, v/v); detection, UV 280 nm.

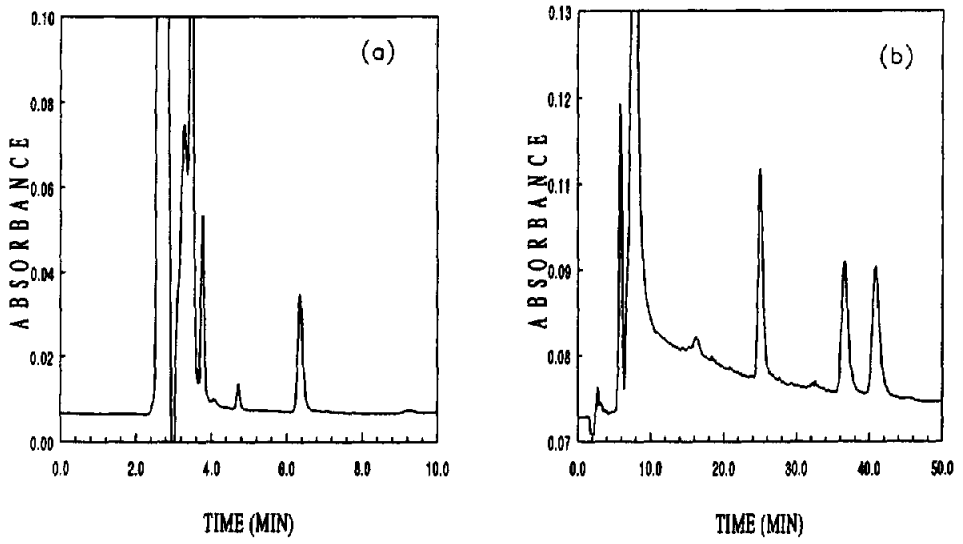

Fig. 4. Chromatograms of drug standards in dog plasma obtained with the switching valve-filter assembly; (a) ibuprofen; (b) estrogens. Conditions the same as in Table 3.

Table 3
Comparison of peak parameters of drugs in dog plasma obtained with switching-valve-filter system

Compound	Parameter	Average ($n = 6$)	S.D.	C.V. (%)
Ibuprofen	Retention time (min)	6.39	0.01	0.23
	Peak area	706000	48100	6.82
	Peak height	36100	2760	7.65
	Peak symmetry	1.04	0.03	2.77
	N	5710	786	13.78
Estradiol	Retention time (min)	25.8	0.38	1.48
	Peak area	329000	17600	5.34
	Peak height	3840	229	5.97
	Peak symmetry	1.12	0.07	5.84
	N	5390	512	9.50
Equilin	Retention time (min)	36.6	0.65	1.77
	Peak area	320000	10900	3.40
	Peak height	2660	133	5.00
	Peak symmetry	1.19	0.06	4.68
	N	5110	471	9.21
Estrone	Retention time (min)	42.6	0.67	1.56
	Peak area	320000	16300	5.10
	Peak height	2460	141	5.74
	Peak symmetry	1.18	0.07	6.06
	N	4650	354	7.60

Conditions: Column, Beckman ultrasphere C_{18}, 250 × 4.6 mm I.D., 5 μm particle size; flow-rate, 0.2 ml/min injection, 1 ml/min analytical; injection volume, 10 μl; ibuprofen: 26 μg/ml; mobile phase, 70% acetonitrile–30% (0.094% triethylamine in water, pH adjusted to 3 with glacial acetic acid); detection, UV 220 nm; estrogens: 10 μg/ml each of estrone, estradiol and equilin; mobile phase, acetonitrile–water (33:67, v/v), UV 280 nm.

4. Conclusion

In this paper, the feasibility of cleaning plasma samples by means of on-line precipitation and filtration using a switching-valve–filter system has been demonstrated. The system parameters evaluated were within acceptable limits with a C.V. below 10% in most cases. Unlike other direct sample injection systems, which often require choosing appropriate pre-columns for the particular analyte(s) under study, this method does not involve the use of pre-columns. The approach can be applied, in a general way, to any analyte system; one only has to choose a specific precipitating agent if common precipitating agents cannot be used. The system is very simple, inexpensive and versatile. It can easily be automated and thus forms a potentially powerful tool for routine analysis and rapid drug screening, especially in a clinical laboratory setting where sample through-put and analysis time are important. In its present set-up the system may not be sensitive enough for ultra-trace analysis. However, after optimization of the injection volume, precipitation time and reagent, the sensitivity could be improved. Although in this work we only demonstrated its usefulness for two analyte systems in one sample matrix, plasma, its application to other analytes in other biological matrices is conceivable. The only problem, in terms of automation, is the need to periodically change filters. However, this may be minimized by using an array of five sets of filters connected in parallel by means of two six-position switching valves such as Rheodyne's Model 7060, one at each end of the filters. This will allow the automatic selection of a new set of filters when one set is determined to be no longer usable. Such an arrangement has been demonstrated by Wyss and Buchelli to be useful for the direct injection of acitretin and 13-*cis*-acitretin using pre-columns [12]. Finally, the system could also be coupled to other on-line sample clean-up devices, such as pre-columns, to increase the clean-up efficiency in situations where the use of filtration alone is inadequate. However, it must be emphasized that when used in this way, the precipitating reagent must not interfere with the operation of the pre-column.

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