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SAMPLE PREPARATION BY SALTS PRECIPITATION AND QUANTITATION BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY WITH UV DETECTION OF SELECTED DRUGS IN BIOLOGICAL FLUIDS*,**

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SUMMARY

A quantitative clean-up procedure for drugs in plasma and urine in preparation for high-performance liquid chromatographic analysis is discussed. Samples are mixed with zinc sulfate, barium hydroxide, and acetonitrile (or methanol). Resultant solutions are clear and free of visible residue. Injections of over 420 samples of treated urine and 120 samples of treated plasma had no noticeable effect on pressure drop and column performance. An examination of the column head indicated no damage or discoloration to the packing. Baselines of the controls show no interference from endogenous compounds for the drugs studied. Precision study using cyclobenzeprine · HCl in plasma and urine by internal standard and external standard methods has within-run and day-to-day variations of under 5%.

Drugs studied in urine and plasma are amiloride · HCl, cyclobenzeprine · HCl, cyproheptadine · HCl, diflunisal, indomethacin, phenylbutazone and sulindac. These drugs are selected for the various functional groups, their binding by proteins and their natural UV absorptivity. Conditions to improve recovery, advantages and limitations are discussed.

INTRODUCTION

In the analysis of drugs in biological fluids, the components of interest readily lend themselves to determination by reversed-phase high-performance liquid chromatography (HPLC). The sample, depending on the drug(s) and its concentration, may entail an elaborate work-up procedure. The factors to be considered when deciding on the sample preparation are concentration, physical and chemical properties of the drug, chromaphore of the drug, biological background of the sample relative to the drug, level of detection required, sensitivity of the detection method chosen, ease and time of technique, and life of the column.

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** The author apologises for the incorrect spelling of cyclobenzaprine · HCl throughout the article.

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Many procedures for sample preparation for reversed-phase HPLC have been reported. They range from the simple direct injection of urine[1,2] and ultra-centrifuged plasma samples[3] to more complicated ones which involve many steps[4–7]. Intermediate methods in ease and time are single extraction by an organic solvent(s)[8–13] and single column elution[14–16]. Some sample preparations, in addition to the removal of endogenous interfering components, also derivatize or hydrolyze the drug to enhance sensitivity by pre- or post-column techniques[17–19].

In this study, a simple, rapid, reliable and inexpensive quantitative method for sample preparation for reversed-phase HPLC is discussed. The method is reproducible and has no degrading effect on column performance used in the absence of a guard column. Other advantages and restrictions are also discussed. This method has been tested on seven drugs, and application to other compounds may be possible. It is hoped that by these model drugs, analysts will be able to decide if the procedure is applicable to their drugs of interest.

EXPERIMENTAL

Equipment

Two Hewlett-Packard (Avondale, PA, U.S.A.) liquid chromatographs were utilized. The HP 1084B was connected to a 79875A variable wavelength detector, a 79850B integrator and a 79841A auto sampler. The HP 1081B was equipped with a LDC Spectromonitor III (Riviera Beach, FL, U.S.A.) UV variable wavelength detector, a 3380A integrator and a 79841A auto sampler. The chromatographic column (30 cm × 3.9 mm I.D.) contained 10 μm μBondapak C_{18} (Waters Assoc., Milford, MA, U.S.A.).

The vortex mixer was a Vortex-Genie Model K-550-G (Scientific Industries, Bohemia, NY, U.S.A.) and the table-top centrifuge was a Dynac II centrifuge Model 103 (Clay Adams, Parsippany, NJ, U.S.A.).

Chromatographic conditions

Injection volume was either 30 μl or 50 μl. Solvents used as mobile phases were aqueous mixtures of acetonitrile and methanol acidified with phosphoric acid, to about pH 2.2. All quantitation was by an external standard method unless otherwise specified. Data obtained for urine and plasma samples were analyzed in triplicate and duplicate, respectively.

Materials

Zinc sulfate heptahydrate crystals (MCB, Cincinnati, OH, U.S.A.) and anhydrous barium hydroxide (Fisher Scientific, Fair Lawn, NJ, U.S.A.) were analytical reagent grade. All drug substances were available as manufactured chemicals by Merck and Co. (Rahway, NJ, U.S.A.).

Acetonitrile and methanol, both glass distilled, were purchased from Burdick and Jackson (Muskegon, MI, U.S.A.). Phosphoric acid 85%, certified ACS grade, was purchased from Fisher Scientific.

Blood from healthy volunteers was mixed with citric phosphate dextrose (Fenwaul, Deerfield, IL, U.S.A.). The plasma thus prepared was frozen in aliquots till use. Urine, obtained from a healthy volunteer, was used without further treatment.

Reagent preparation

Saturated barium hydroxide was prepared by adding anhydrous barium hydroxide in excess to water. The solution was filtered as needed. Aqueous zinc sulfate 15% was prepared on an anhydrous weight basis.

Sample preparation

Urine. To 2.0 ml of urine, 0.1 ml of 15% (w/w) zinc sulfate was added. The sample was then vortex-mixed. After 5 min, 0.4 ml of saturated barium hydroxide was added and vortex-mixed. After another 5 min, a suitable volume of organic solvent was added and vortex-mixed. The sample was then centrifuged at 1180 *g* for 5 min. The supernatant was ready for direct chromatographic analysis.

Plasma. To 1.0 ml plasma, 0.1 ml of 15% (w/w) zinc sulfate was added and then vortex-mixed. After a 5-min interval, 0.2 ml of saturated barium hydroxide was added. The sample was vortex-mixed and 5 min later, a suitable volume of organic solvent was added. The sample was then vortex-mixed and centrifuged at 1180 *g* for 5 min. The supernatant was ready for direct chromatographic analysis.

Precision study

Samples were prepared as described under *Sample preparation.* For the internal

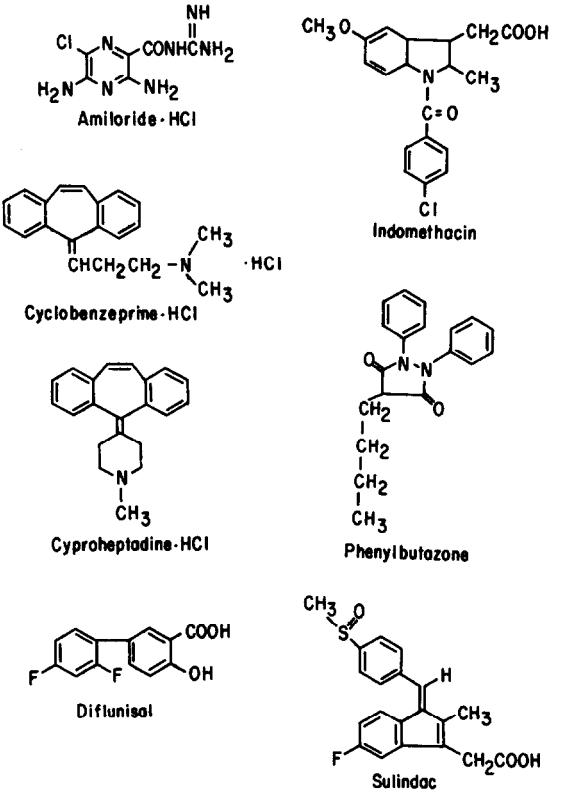


Fig. 1. Drugs that were studied in the salts precipitation method in urine and plasma.

TABLE I

RESULTANT pH OF PLASMA SAMPLES

The following volume of organic solvent was mixed with 2 ml plasma (pH 7.9), 0.5 ml zinc sulfate, 0.5 ml barium hydroxide and adjusted to 5 ml with water.

Volume of organic solvent (ml)	*Final pH*	
	Acetonitrile	*Methanol*
0	6.6	6.6
0.5	6.5	6.5
1.0	6.3	6.4
2.0	6.0	6.2

standard method, cyclobenzaprine · HCl was added at 7.5 μg/ml of plasma and diflunisal at 7.4 μg/ml of urine as internal standards. Internal and external standard curves were run for each separate set of data.

RESULTS AND DISCUSSION

Zinc sulfate and barium hydroxide have been used for the termination of enzymatic reactions[20]. An application of this concept for HPLC has been made to the analysis of furaltadone in biological fluids in a pharmacokinetic study in goats[21]. In the present study, the salt combination is modified by the addition of acetonitrile or methanol and evaluated for seven drugs (Fig. 1) in human biological fluids.

The procedure described herein is rapid and inexpensive. The number of samples that can be prepared in 20 min is limited only by the number of fixed volume additions to tubes in 5 min. These simple steps render robotic control a possibility. The resultant supernatant is free of visible residue, and direct reversed-phase HPLC analysis indicates no interference from endogenous materials for the drugs studied. All samples are analyzed without a guard column. Injections of over 420 urine samples and 120 plasma samples for more than three months resulted in no noticeable

TABLE II

RESULTANT pH OF URINE SAMPLES

The following volume of organic solvent was mixed with 4 ml urine (pH 6.2), 1 ml zinc sulfate, 1 ml barium hydroxide and adjusted to 8 ml with water.

Volume of organic solvent (ml)	*Final pH*	
	Acetonitrile	*Methanol*
0	6.5	6.5
0.25	6.4	6.5
0.5	6.4	–
1.0	6.4	6.4
1.5	6.3	–
2.0	6.2	6.3

TABLE III

RESULTANT pH AND RECOVERY FROM pH-ADJUSTED URINE

Four ml urine was mixed with 0.5 ml drug in acetonitrile, 1 ml zinc sulfate and 1 ml of barium hydroxide. Initial pH was adjusted by 1 *M* hydrochloric acid or 1 *M* sodium hydroxide.

Initial pH	*Final pH*	*Cyclobenzeprine · HCl recovered (%)*
5.2	6.1	97.5
6.1	6.1	98.9
6.8	6.2	96.9
7.7	6.3	97.7

effect on the performance or pressure difference of a column. An examination of the exposed column head indicated no loss of packing or discoloration.

A restriction of this method is compatibility with mobile phases. Metal residues are present in the sample and mobile phases which contain components that form insoluble salts with zinc or barium have to be avoided. Similarly, columns with a previous history of such components should be thoroughly washed before use.

The resultant urine and plasma samples are buffered. To illustrate the buffering effect of the reagents, urine and plasma samples were mixed with the same ratio of

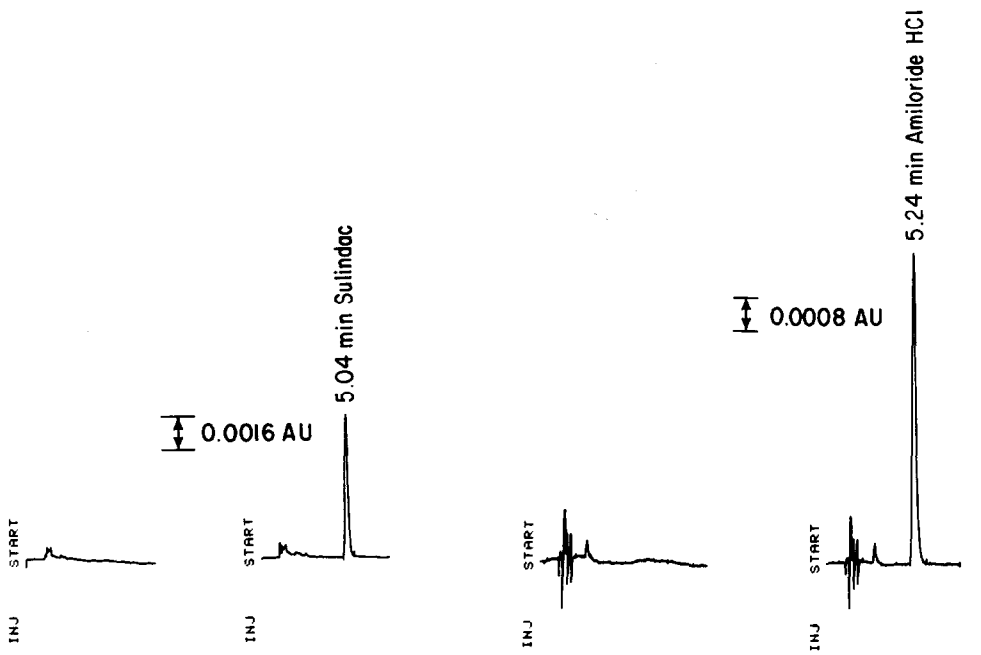


Fig. 2. Control and sulindac in plasma with HPLC conditions: 42% acetonitrile in 0.1% phosphoric acid, 40°C oven, 2 ml/min flow-rate and 325 nm detector for 72 ng in 30 μl injected.

Fig. 3. Control and amiloride · HCl in plasma with HPLC conditions: 12% acetonitrile in 0.1% phosphoric acid, 30°C oven, 2 ml/min flow-rate and 360 nm detector for 60 ng in 50 μl injected.

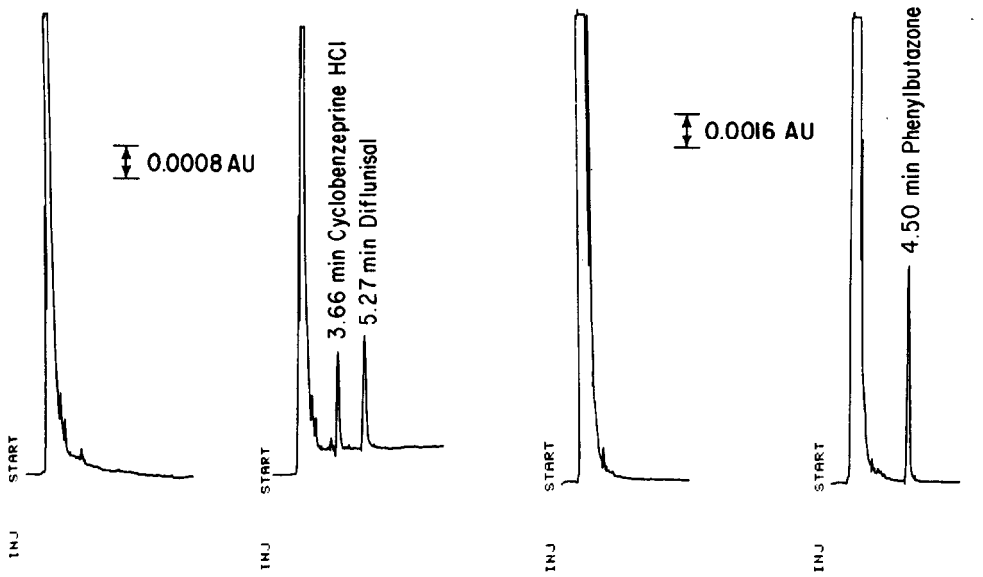


Fig. 4. Control and analytes in plasma with HPLC conditions: 55% acetonitrile–methanol (3:1) in 0.1% phosphoric acid, 40°C oven, 2 ml/min flow-rate and 275 nm detector for 29 ng cyclobenzeprine · HCl and 60 ng diflunisal in 50 μl injected.

Fig. 5. Control and phenylbutazone in urine with HPLC conditions: 60% acetonitrile in 0.1% phosphoric acid, 40°C oven, 2 ml/min flow-rate and 270 nm detector for 300 ng in 30 μl injected.

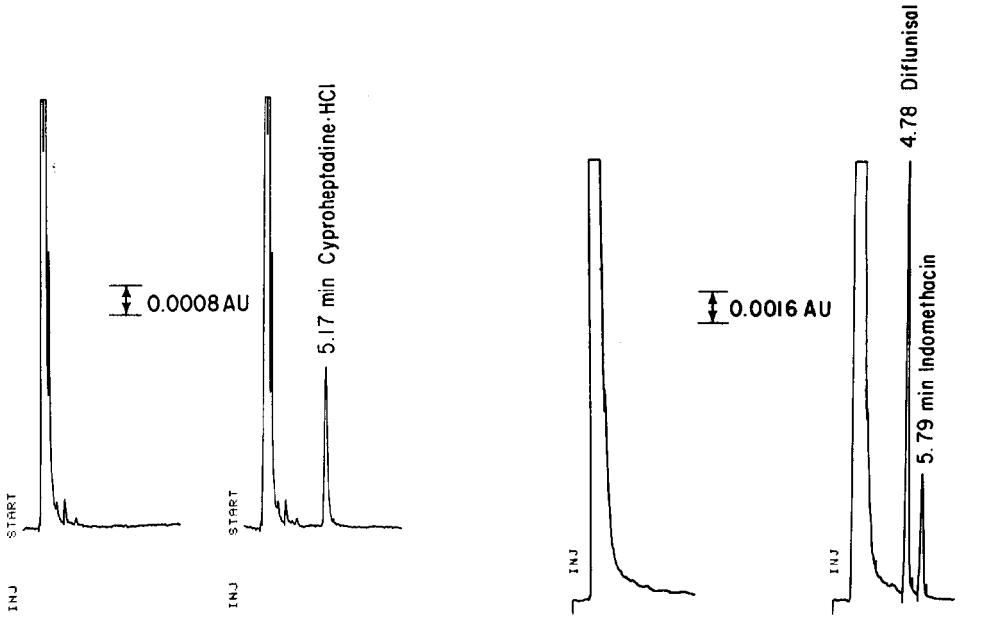
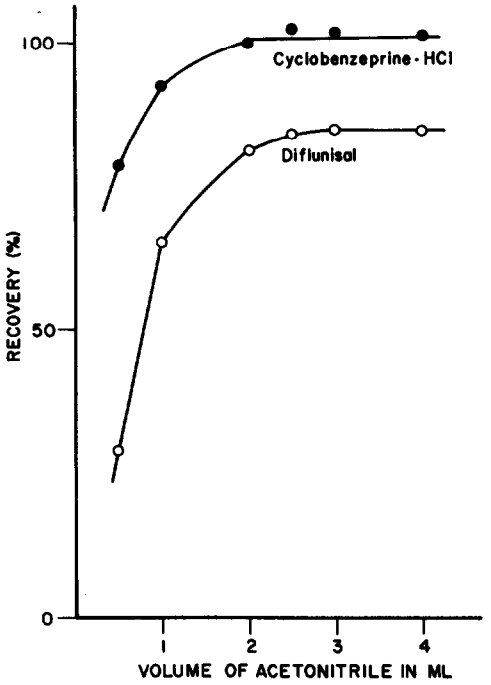


Fig. 6. Control and cyproheptadine · HCl in urine with HPLC conditions: 37% acetonitrile in 0.1% phosphoric acid, 40°C oven, 2 ml/min flow-rate and 285 nm detector for 65 ng in 50 μl injected.

Fig. 7. Control and analytes in urine with HPLC conditions: 60% acetonitrile–methanol (7:3) in 0.1% phosphoric acid, 30°C oven, 2 ml/min flow-rate and 254 nm detector for 42 ng indomethacin and 113 ng diflunisal in 30 μl injected.



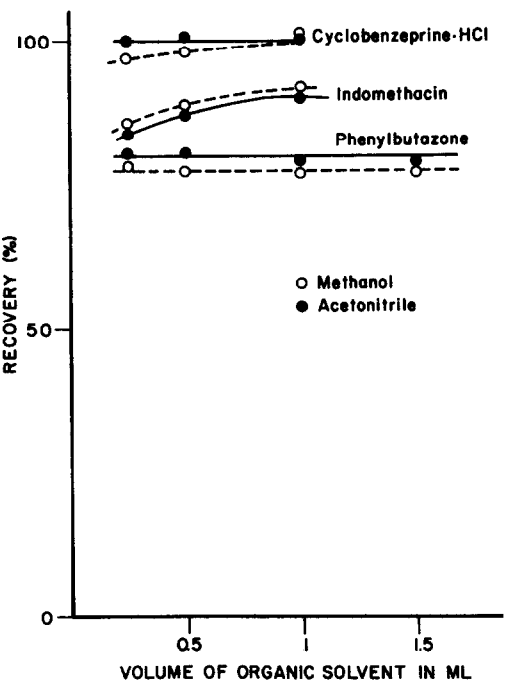


Fig. 8. Recovery *vs.* volume of acetonitrile for analytes added to plasma.

Fig. 9. Recovery *vs.* volume of organic solvent for analytes added to urine.

reagents and various volumes of organic solvent. The final constant volume was adjusted with water. Tables I and II indicate fairly similar apparent pH for both urine and plasma samples. The resultant solution is close to neutral pH. The slight decrease in apparent pH with increase in organic solvent is due to the organic solvent. The resultant apparent pH of the biological fluids preparation as described in the Experimental section is elevated by no more than 1 pH unit because of the higher ratio of barium hydroxide. No difference is observed in the control chromatograms for plasma and urine using either ratio of reagents.

In a separate experiment, urine was adjusted to a pH range between 5 and 8 with hydrochloric acid or sodium hydroxide. Cyclobenzeprine · HCl in 0.5 ml of acetonitrile and 1.0 ml each of the reagents was added to the pH-adjusted urine. Recovery and final apparent pH data are in Table III. The neutral pH of the final solution is maintained. For compounds stable only at acid or alkaline pH, the buffered resultant sample solution offers only a short storage life.

The drugs for this study cover broad therapeutic classes and a range of functional groups. In addition, they are selected because of the natural UV absorptivity and the binding property by protein. The endogenous material removed is independent of the amount of organic solvent added as observed on the control chromatograms. Chromatograms for plasma and urine samples with known amounts of analyte added and their control are shown on Figs. 2 to 7. Baselines of the controls show no interference from endogenous compounds. In general, recovery of the drugs studied

TABLE IV

PRECISION DATA BY INTERNAL STANDARD METHOD

Details are described under *Precision study*. S.D. = standard deviation; C.V. = coefficient of variation; n = number of determinations; $\bar{X}$ = mean value.

	μg/sample added	*μg/sample detected*	*S.D. (μg/sample)*	*C.V. (%)*	*n*
Indomethacin in urine					
Day 1	9.6	9.46	0.35	3.7	8
Day 2	9.6	9.46	0.21	2.2	8
	Net $\bar{X}$ =	9.46	C.V. =	2.9	
Indomethacin in plasma					
Day 1	11.46	11.35	0.27	2.3	6
Day 2	11.46	10.86	0.49	4.5	7
	Net $\bar{X}$ =	11.09	C.V. =	4.2	

in urine and plasma increases with increasing organic solvent until recovery plateaus. For some drugs, the plateau is reached with a lower amount of organic solvent. Figs. 8 and 9 illustrate the pattern trend using a few representative drugs in plasma and urine. No obvious correlation appears to exist between functional groups and recovery for the drugs tested. Recovery is complete for the tricyclics tested. However, with drugs highly bound to protein[22–24], a lower recovery plateau is observed. These drugs act as model compounds and it is hoped that by the examples and the discussion on advantages and restrictions, the analysts will have a feel for optimizing a possible assay for their drug of interest using this procedure.

To evaluate precision, a known amount of analyte was added to urine and

TABLE V

PRECISION DATA BY EXTERNAL STANDARD METHOD

Details are described under *Precision study*.

	μg/ml added	*μg/ml detected*	*S.D. (μg/ml)*	*C.V. (%)*	*n*
Indomethacin in urine					
Day 1	3.05	2.80	0.10	3.4	8
Day 2	3.05	2.76	0.05	1.7	8
	Net $\bar{X}$ =	2.78	C.V. =	2.7	
Cyclobenzeprine · HCl in plasma					
Day 1	2.81	2.20	0.01	0.4	5
Day 2	2.81	2.21	0.03	1.3	6
	Net $\bar{X}$ =	2.21	C.V =	0.9	
Diflunisal in plasma					
Day 1	3.96	1.23	0.03	2.2	5
Day 2	3.96	1.24	0.03	2.3	6
	Net $\bar{X}$ =	1.24	C.V. =	2.2	

plasma for an intra- and an inter-run comparison using external standard and internal standard methods. All samples contained 0.5 ml acetonitrile and recovery was not optimized. Optimum organic solvent level has been shown to vary from compound to compound. Standard curves for both internal and external standard methods had regression constants of 0.999 or better. Data are shown in Tables IV and V. The coefficient of variation observed for within run or between runs in plasma and urine ranges from 0.4% to 4.5%.

Detection for drugs in this study is confined to UV absorbance. In this case, the sample preparation procedure is favored for drugs that absorbed at relatively higher wavelength and are less polar. Use of UV detection may, in some cases, be limiting for a rigorous pharmacokinetic study because of sensitivity. Enhancement of sensitivity by employing either fluorescent or electrochemical detectors using this procedure is possible. The ease and time of preparation is balanced by the biological background of the sample and level of sensitivity required. However, if most of the endogenous components are eliminated from the sample, injection volume may be increased with a concomitant increase in sensitivity.

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