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Use of guanidine hydrochloride and ammonium sulfate in comprehensive in-line sorption enrichment of xenobiotics in biological fluids by high-performance liquid chromatography

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ABSTRACT

A novel approach has been developed for direct injection of physiological fluids on an in-line extraction pre-column followed by column switching in order to introduce the adsorbed xenobiotic onto the analytical column. The physiological fluid is pre-treated with guanidinium solution in water (200 μl of fluid plus 300 μl of a reagent containing 8.05 *M* guanidinium and 1.02 *M* ammonium sulfate) in order to denature protein binding sites and to serve as a universal solvent for a divergent range of polar to non-polar xenobiotics in a hydrophilic medium. A 0.5 *M* ammonium sulfate solution (500 μl) is used as a pre- and post-flush reagent for the extraction pre-column (30 mm × 2.1 mm I.D.). The pre-flush reagent prepares the sorbent environment of the C_{18} pre-column for the hydrophobic retention of analytes. The post-flush reagent flushes non-retained sample proteins and salts to waste prior to switching the pre-column in-line with the analytical column. Universal chromatographic conditions for the analytical phase allows elution of a range of polar to non-polar xenobiotics within 20 min from an end-capped C_8 silica analytical column (250 mm × 4.6 mm I.D.). This is effected by a linear gradient from a binary system consisting of solvent A (0.05 *M* KH_2PO_4) and solvent B (acetonitrile–isopropanol, 80:20, v/v).

INTRODUCTION

Substantial effort in the development of HPLC technology has been directed to the automation and direct injection of physiological fluids onto an in-line extraction pre-column followed by column switching in order to introduce the adsorbed xenobiotic sample onto the analytical column [1–10]. The latest variation of these techniques includes the use of sodium dodecyl sulfate (SDS) which forms micelles with plasma proteins and allows adsorption of the compounds of interest on the pre-column [11–13].

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In spite of the vast information available on all sorts of HPLC applications, we have found it necessary to develop our own strategy for universal HPLC conditions in order to cope with the analytical demands experienced in our relatively large routine toxicology service laboratory. Since we believe that our HPLC methodology is sufficiently versatile so as to fulfill a first-line analytical role in many laboratories we would like to describe its details in this paper. As a first approach, it was reasoned that a reagent for pretreating the sample (particularly serum) should release xenobiotics from their protein binding sites and serve as a universal solvent for a divergent range of polar and non-polar xenobiotics. For this purpose, guanidinium was chosen. This salt is well known as a protein denaturing

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agent as well as a universal solubilizing agent for polar and non-polar substances [14]. It was also necessary to develop a solution with which the plasma proteins can be flushed from the pre-column, while the hydrophobic interaction of the xenobiotics adsorbed on the pre-column is maintained in order to allow subsequent quantitative analysis by switching to the analytical column. According to the Hofmeister series of hydrophobic bond potentiating cations and anions [15], ammonium sulfate was chosen as the most suitable inorganic salt for this purpose.

Except for the above requisites, it must be realized that the development of these methods is not easily explained by further theoretical reasoning. The Experimental section of this paper therefore describes the details of the optimised compromise of HPLC conditions, while the Results section illustrates application to a large range of xenobiotics with divergent chemical and physical properties.

EXPERIMENTAL

Equipment

The instrument used was supplied by Hewlett Packard (Waldbron, Germany) and consisted of the following components: HP 1090 M liquid chromatograph with a binary DR5 solvent delivery system and manual valve injector, HP 1040 diode-array detector, HP 79994A workstation, HP 310 SPU processor with colour monitor, HP 9153C 20 MB Winchester disc drive, HP 2225A thinkjet printer and HP 7440A X–Y plotter.

The plumbing configuration of the in-line column in the sample load and extraction phase is shown in Fig. 1 together with the plumbing configuration of the analytical phase during which the adsorbed xenobiotics are resolved. The analytical column (250 mm × 4.6 mm I.D.) is packed with end-capped C_8 bonded silica of 5 μm particle size (Whatman, Maidstone, UK) while the guard column (75 mm × 2.1 mm I.D.) is packed with Pellicular C_{18} bonded silica (Whatman). The extraction pre-column is packed with preparative-grade C_{18} bonded silica of 40 μm particle size (Analytichem International, Harbor

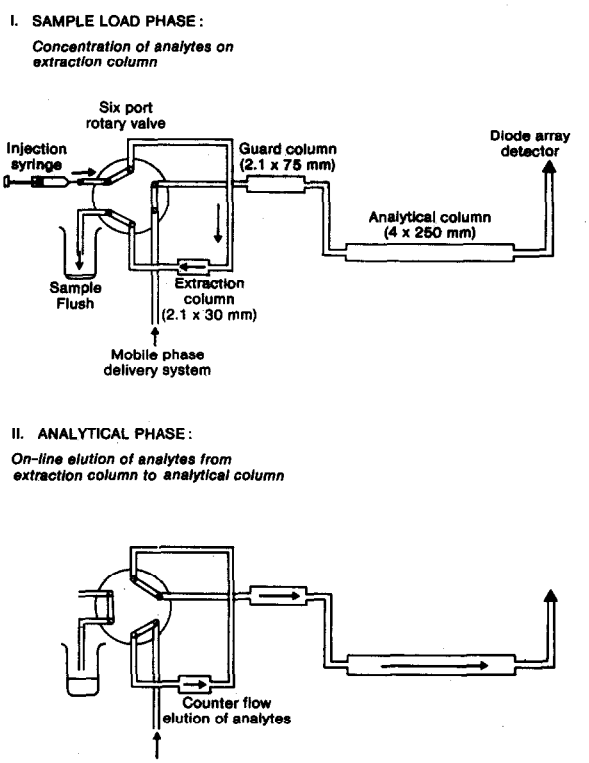

Fig. 1. Configuration of the in-line extraction pre-column. In the sample load phase the analytes are retained on the sorbent of the pre-column, while the sample matrix is flushed to waste. In the analytical phase the analytes are eluted from the pre-column onto the analytical column by the mobile phase. Resolved components are detected by the diode-array detector in terms of UV absorption spectrum and chromatographic retention time.

City, CA, USA). The connecting stainless-steel tubing has an internal diameter of 0.1 mm.

Chemicals and solvents

Xenobiotic standards were obtained from pharmaceutical manufacturing companies or from Sigma (St. Louis, MO, USA). Where necessary, they were recrystallized in order to obtain chromatographic purity.

Acetonitrile, isopropranol and methanol were HPLC grade purchased from Burdick and Jackson (Muskegon, MI, USA). Ultrapure guanidinium (guanidine hydrochloride) was purchased from Bethesda Research Chemicals (Bethesda, MD, USA) for routine use. Analytical-grade guanidinium obtained from Merck (Darmstadt, Germany) contained significant UV-absorbing

impurities (*vide infra* Fig. 3). Potassium dihydrogenphosphate and ammonium sulfate were analytical grade purchased from Merck. HPLC-grade water was prepared from distilled deionized water by filtration through a Millipore (Bedford, MA, USA) system.

Sample preparation

Dessicated xenobiotic standards were accurately weighed out (10 mg) and dissolved in methanol. Appropriate volumes of each dissolved xenobiotic were mixed, taken to dryness under nitrogen and then dissolved in 50 μl methanol to which 10 ml of commercial drug-free serum (Q-pack Hyland Diagnostics, Deerfield, IL, USA) was added and incubated for 2 h at 37°C. Tables I and II give the final concentrations of each xenobiotic analyzed.

The injection sample for biological fluids, *i.e.* serum, urine, cerobrospinal fluid and gastric aspirate, consisted of mixing 200 μl of the fluid with 300 μl of the pre-treatment reagent. The latter solution consisted of 8.05 *M* guanidinium and 1.02 *M* ammonium sulfate in water.

Chromatographic conditions

The sample load phase consisted of by injecting 500 μl of 0.5 *M* ammonium sulfate onto the in-line extraction column with the six-port rotary valve in the sample load position (Fig. 1). This was followed by sample injection; 500 μl of 0.5 *M* ammonium sulfate were then injected to elute plasma proteins from the in-line extraction column. The latter had an exclusion volume of 112 μl. The analytical phase was initiated by direct activation of the six-port rotary valve (Fig. 1).

The operating conditions in the analytical phase were programmed at a flow-rate of 1.5 ml/min starting with mobile phase A (0.05 *M* KH_2PO_4) to which 10% mobile phase B (acetonitrile–isopropanol, 80:20, v/v) was added after 0.01 min. This mixture was maintained for 1 min after which a linear gradient of mobile phases A and B were formed which ended in 70% B after 20 min. The temperature of the column compartment was maintained at 50°C.

The diode-array detector was programmed to detect xenobiotic peaks at seven wavelengths ranging from 200 to 280 nm. A UV absorbtion profile ranging from 200 to 400 nm of each peak was also obtained at its leading edge, peak and trailing edge in order to check for co-elution of xenobiotics.

RESULTS

In order to demonstrate the effect of the pre-treatment reagent on the extraction efficiency of xenobiotics from serum by in-line adsorption it was necessary to select a standard mixture of xenobiotics with wide-ranging physical properties in terms of polarity, water solubility and protein binding. The mixture was dissolved in commercial drug-free serum as outlined in the Experimental section.

Table I lists the xenobiotics in the standard mixture together with their concentrations in serum. Tryptophan, which is present in serum, is also listed in Table I. It elutes relatively early at 4.2 min and can therefore be used as an internal standard (*vide infra* Fig. 3).

Fig. 2 shows the elution profile of the standard xenobiotic mixture when it was chromatographed without and with the pre-treatment reagent. The peak heights of salicylic acid (peak 2), propoxyphene (peak 4) and indomethacin (peak 12) increased remarkably when the pre-treatment reagent was added to the serum. The percentage increase in extraction efficiency for each xenobiotic is given in Table I. It ranges from 0% for butobarbitone to 100% for propoxyphene. Except for Coomassie blue and salicylic acid the final recoveries, when compared to standard sample loop injection, ranged from 89% to 99% which illustrates the quantitative properties of the in-line pre-column.

Fig. 3 shows a chromatogram of drug-free serum obtained from a local volunteer and treated with guanidinium before injection. The reagent peaks are UV-absorbing impurities in the guanidinium. They increase dramatically when analytical-grade guanidinium is substituted for the ultrapure preparation. The endogenous tryptophan peak is followed by a number of small in-

TABLE I

INCREASE IN THE EXTRACTION EFFICIENCIES OF XENOBIOTICS WHEN GUANIDINIUM IS ADDED IN A 2:3 RATIO TO SERUM AS A PRE-TREATMENT REAGENT

The sample load sequence is as in Fig. 2. The final recoveries from the pre-column with the pre-treatment reagent were calculated from values obtained when injections were made via a sample loop.

Peak No. in Fig. 2	Xenobiotic	Serum concentration (μg/ml)	Retention time (min)	Increase in extraction efficiency (%)	Final recovery (%)
1	Tryptophan	–	4.2	17.5	–
2	Salicylic acid	15	5.2	80.9	74.2
3	Caffeine	5	6.1	6.2	97.9
4	Propoxyphene	5	6.9	100	99.0
5	Lignocaine	10	8.0	8	99.4
6	Sulphamethoxazole	10	8.9	31.4	89.6
7	Quinine	5	9.8	16.6	98.8
8	Butobarbitone	20	10.9	0	98.4
9	Carbamazepine	5	11.7	13.8	98.4
10	Desipramine	10	13.3	42.8	90.2
11	Coomassie blue	20	14.2	19.5	66.9
12	Indomethacin	5	15.3	80.0	91.5
13	Prazepam	5	16.9	20.9	90.3

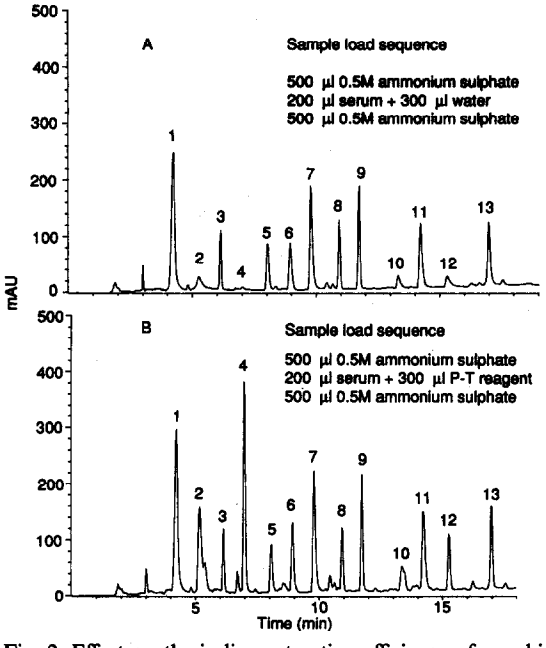

Fig. 2. Effect on the in-line extraction efficiency of xenobiotics from serum when water (A) is substituted for the serum pretreatment (PT) reagent (B). The xenobiotics which correspond to each numbered peak are given in Table I together with their serum concentration and retention time. The effect of the PT reagent is most remarkable for salicylic acid (peak 2), propoxyphene (peak 4) and indomethacin (peak 12).

dole-related peaks (*i.e.* their UV spectra resemble that of tryptophan closely). It has previously been shown that the specific conditions of chromatography described here will resolve metabolites of tryptophan [9]. Heptanophenone elutes between 18 and 19 min and we consider it a convenient substance for an internal standard. Only a few very non-polar substances elute at a later time (*vide infra* Fig. 4A). The presence of substance X at a retention time of 12.2 min is reported here only for the sake of completeness. It is an

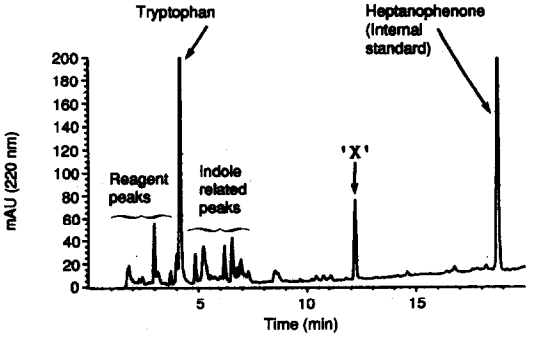

Fig. 3. Chromatogram of drug-free serum obtained from a human volunteer. The sample was treated with guanidinium and loaded as shown in Fig. 1.

unknown substance of dietary origin and is not found in commercial drug-free serum. Its structure and other properties will be reported in a separate communication.

It is clear from Fig. 3 that the time between the elution of tryptophan at 4.2 min and heptanophenone at 18.6 min forms a relatively clean analytical window in which substances of interest can be eluted. Fig. 4 shows four chromatograms of mixtures of eight to eleven xenobiotics dissolved in serum and pre-treatment reagent as examples which were used for calibration purposes and to construct a reference library. The xenobiotics are listed in Table II according to the peak numbers shown in Fig. 4.

Inspection of the retention times of the xenobiotics listed in Table II shows that none of the 39 had an exactly similar value. Some of them are, however, very close, for example malathion at 16.31 min (A), flufenamic acid at 16.26 min (B) and prazepam at 16.92 min (C). An increase in the number of xenobiotics analyzed will therefore obviously result in the co-elution of some. In such a case the additional discriminative ability of the diode-array detector will be decisive.

The concentrations of the xenobiotics used to produce the chromatograms shown in Fig. 4 are also listed in Table II. In addition, six-point calibration curves over the concentration ranges shown in Table II have been constructed for each xenobiotic using heptanophenone as internal standard. All the curves could be fitted with a straight line or a second-degree polynomial with correlation coefficients (r) equal to 0.999–1.000.

Fig. 5 shows analyses of samples from a patient admitted to the hospital after organophosphate poisoning. Serum, urine and gastric aspirate were analyzed soon after adminission. Malathion (7 μg/ml) and chlorpyriphos (1.38 μg/ml) were found in the serum. Their concentrations were obviously higher in the gastric aspirate while they were still undetectable in the urine 2 h

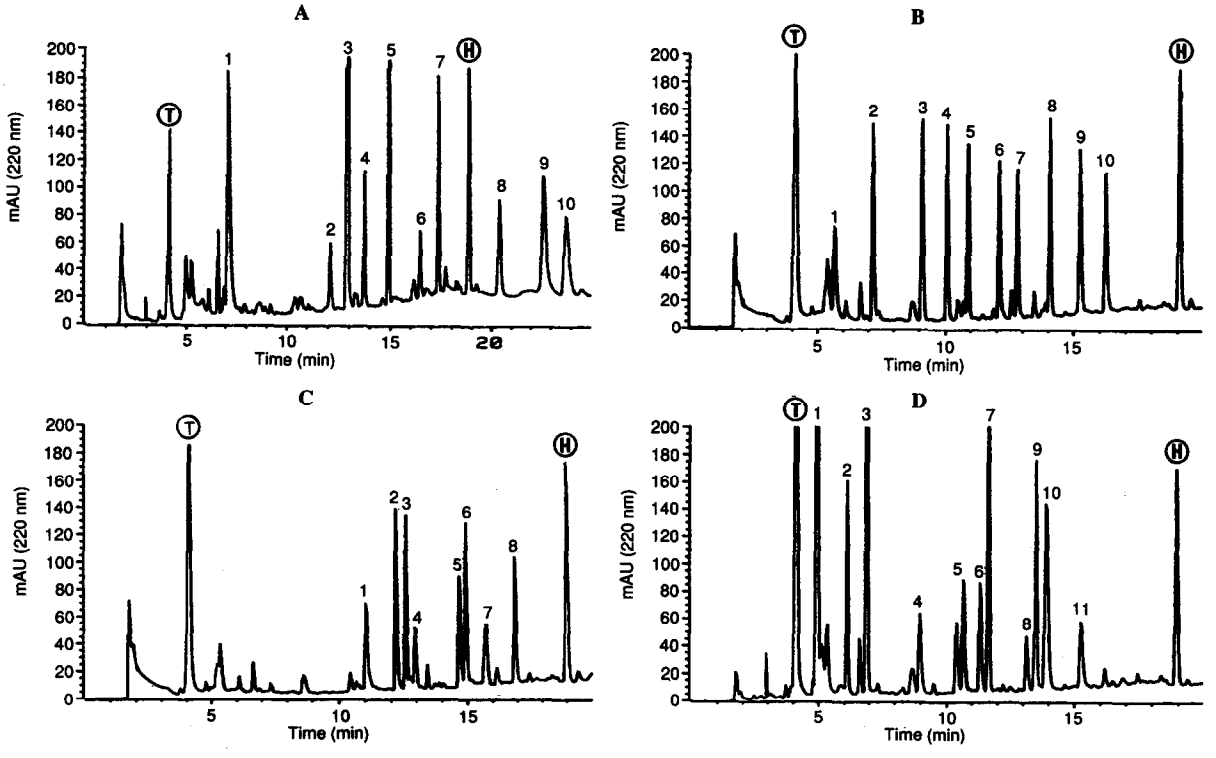

Fig. 4. Chromatograms of four standard xenobiotic mixtures A, B, C and D dissolved in serum. T and H denote tryptophan and heptanophenone, respectively, while the numbered peaks correspond to a xenobiotic as given in Table II.

TABLE II

FOUR SETS OF XENOBIOTIC STANDARDS (A, B, C AND D) DISSOLVED IN SERUM AND CHROMATOGRAPHED IN ORDER TO CALIBRATE THE ANALYTICAL SYSTEM

Chromatograms are shown in Fig. 4.

Xenobiotic	Peak No. in Fig. 4		Retention time (min)	Concentration (μg/ml)	
				Of peak shown in Fig. 4	Range investigated[a]
Strychnine	A	1	7.13	5	1–30
Formothion		2	12.09	10	2–15
Carbaryl		3	12.89	5	1–30
Paraoxon		4	13.73	5	1–30
Azinphos		5	14.8	5	1–30
Malathion		6	16.31	5	2–30
Chlorvinphos		7	17.34	5	1–30
Chlorpyriphos		8	20.38	5	1–30
Ethylbromphos		9	22.62	5	1–30
Prothiophos		10	23.74	5	1–30
Chlorothiazide	B	1	5.67	3	1–24
Barbitone		2	7.21	15	5–120
Allobarbitone		3	9.12	15	5–120
Brallobarbitone		4	10.07	15	5–120
Butobartitone		5	10.90	15	5–120
Pentobarbitone		6	12.12	15	5–120
Secobarbitone		7	12.82	15	5–120
Thiopentone		8	14.10	15	5–120
Indomethacin		9	15.27	3	1–24
Flufenamic acid		10	16.26	3	1–24
Bromazepam	C	1	11.05	2	1–20
Oxazepam		2	12.21	2	0.5–20
Lorazepam		3	12.57	2	0.5–20
Chlordiazepoxide		4	12.96	2	0.5–20
Midazolam		5	14.69	2	0.5–20
Diazepam		6	14.95	2	0.5–20
Medazepam		7	15.79	2	0.5–20
Prazepam		8	16.92	2	0.5–20
Paracetamol	D	1	4.93	40	10–180
Caffeine		2	6.12	4	1–18
Propoxyphene		3	6.90	4	1–18
Cocaine		4	8.97	4	1–18
Chloramphenicol + succinate ester		5	9.92	4	1–18
Thioridazine		6	11.33	4	1–18
Carbamazepine		7	11.66	4	1–18
Diphenhydramine		8	13.14	4	1–18
Griseofulvin		9	13.51	4	1–18
Clothiapine		10	13.92	4	1–18
Dipipanone		11	15.25	4	1–18

[a] The minimum and maximum values are the start and end points of six-point calibration curves all of which could be fitted by a straight line or a second-degree polynomial with r values of 0.999–1.000.

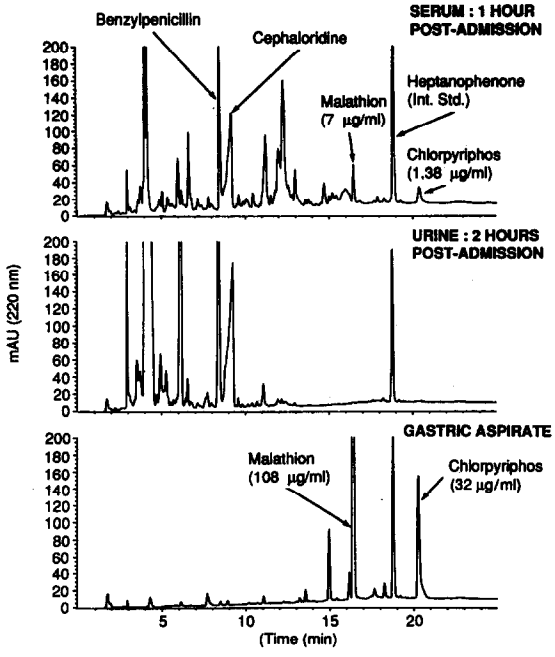

Fig. 5. Series of chromatograms representing different samples taken from a patient after ingestion of organophosphates.

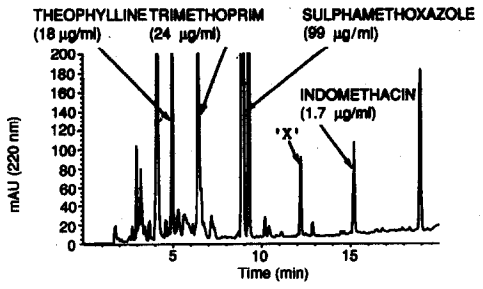

Fig. 6. Chromatogram of the serum of a patient with a history of taking several medicines simultaneously.

post-admission. Fig. 5 also demonstrates the ability of the method to screen for a range of xenobiotics. Much more polar molecules like benzylpenicillin and cephaloridine, which were administered to the hospitalized patient, were detected in the same analysis.

Fig. 6 shows another example of analysis of a patient's serum where the ingestion of theophylline, trimethoprim, sulphamethoxazole and indomethacin could be confirmed together with their respective concentrations.

It is clear from Figs. 5 and 6 that the application of the technology to analytical toxicology is effective and that qualitative and quantitative results are obtained as early as 30 min after receiving the sample.

DISCUSSION

The major objectives of this study were to develop HPLC methodology characterized by quantitative in-line adsorption trapping of xenobiotics and the flushing to waste of plasma proteins before column switching to the analytical phase (Fig. 1) and universal chromatographic conditions for resolution of a range of xenobiotics with divergent chemical and physical properties. For these purposes guanidinium was used as a pre-treatment reagent for protein denaturation and as a universal solvent for polar and non-polar xenobiotics while ammonium sulfate was employed as a hydrophobic bond promoting pre- and post-flush reagent.

From Fig. 3 and the other chromatograms shown, it is clear that the post-flush step in the sample loading phase is effective in removing serum proteins from the sample before switching to the analytical column. Further, when the results shown in Table I are taken together, the percentage increase in extraction caused by the pre-treatment reagent can be divided into three catagories: those with zero to modest increase (0–15%) like caffeine, lignocaine, butobarbitone and carbamazepine; those with an intermediate increase (15–50%) like tryptophan, quinine, sulphamethoxazole, desipramine, Coomassie blue and prazepam; and those with a high increase (>50%) such as salicylic acid, propoxyphene and indomethacin.

The intermediate increase in the extraction efficiency of Coomassie blue (Table I) is also remarkable since it binds so avidly to proteins that it is used as a general stain. The pre-treatment reagent is thus demonstrated to release xenobiotics from both high capacity and high affinity protein binding sites.

We have studied the chromatographic behaviour of a relatively large number of xenobiotics

(Fig. 4 and Table II). The list is, however, by no means complete. To date more than one hundred xenobiotics have been calibrated for their quantitation in serum. It is of special value to us for handling the urgent, non-routine request for quantitation of a xenobiotic in a biological fluid. Provided that the requirements for the concentration limits are met, no special methods development in terms of sample preparation or chromatographic conditions need to be done. A further advantage lies in the relatively small volume (200 μl) of serum needed from paediatric patients for analytical toxicology analysis. The latter group of patients constitutes about 50% of all the toxicological requests received by us.

Finally, in our hands, the system proved extremely robust. We experienced an acceptable durability in terms of the number of injections (200–500) which could be made before it became necessary to change a column.

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