Journal of Chromatography, 381 (1986) 63—74
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3179

USE OF ENZYMATIC SOLUBILIZATION OF TISSUES AND DIRECT INJECTION ON PRE-COLUMNS OF LARGE VOLUMES FOR ANALYSING BIOLOGICAL SAMPLES BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY

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(First received January 6th, 1986, revised manuscript received March 21st, 1986)

SUMMARY

A pre-column technique, which can be used to introduce all types of biological samples into high-performance liquid chromatographic systems, is described. Fluids are injected directly, whereas faeces are homogenized and centrifuged and tissue samples are solubilized by the enzyme subtilisin A. Acetonitrile is added to all samples (no precipitation of proteins is seen), fluids (10%), solubilized tissue (25%) and faeces (20%), to obtain better wettability of the packing and to counteract binding. Samples of up to 50 ml are injected onto a 6 × 4 mm I.D. pre-column from which the compounds are backflushed onto the analytical column. Different packing materials for the pre-column have been used; LiChroprep RP-18 gave the best results. This system has been used for a series of different drugs, using different analytical columns and different detectors. Both gradient and isocratic elution have been used. High recoveries, good reproducibility and low detection limits are seen for routine analysis. For metabolic work, a very large enrichment factor is obtained.

INTRODUCTION

Many drugs are highly active and thus given in low doses. Since they are also strongly bound to proteins, analysis of these drugs is not a trivial problem. A time-consuming and often complicated work-up and enrichment procedure is usually needed before a chromatographic technique can be used to separate the compounds. To circumvent the problems with the extraction techniques, column switching with direct injection of fluids on a pre-column was introduced. The pre-columns used are usually short (< 30 mm long) columns packed with a reversed-phase material [1—13], normal-phase material [14, 15],

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a material for ion exchange [16, 18] or exclusion chromatography [19—21]. The samples analysed by high-performance liquid chromatography (HPLC) with this technique are liquid samples (e.g. serum, plasma, urine, milk and water). Also, supernatant from incubation with isolated hepatocytes or microsomes from rats has been analysed in this way [1, 2]. The injected volume is generally much less than 1 ml for serum samples. A general description of the column-switching technique is given in refs. 22 and 23.

We were interested in using the pre-column technique for (i) routine analysis of different drugs in serum, (ii) analysis of drugs and metabolites in serum, urine, faeces and tissue samples from toxicological studies, and (iii) isolation of unknown metabolites in biological fluids and tissues from rats after dosing with new substances. Point (iii) requires large injection volumes because some metabolites are present in small amounts.

The system described by Kronbach [1] and Voelter et al. [2] was chosen because it is a simple manual system. By using this system, it has been possible to develop a general procedure for the injection of large volumes of all types of biological samples (e.g. fluids, faeces and tissues).

EXPERIMENTAL

Instrumentation

HPLC system I consisted of a Spectra-Physics (Houston, U.S.A.) SP 8700 gradient pump, a Rheodyne (Berkeley, U.S.A.) Model 7125 injection valve with a pre-column instead of the sample loop, and a Hewlett-Packard (Waldbronn, F.R.G.) HP 1040 A photo-diode array detector. The detector was mastered by a HP-85B computer (Hewlett-Packard, Corrallis, U.S.A.) with a dual disc drive (HP-9121D, Hewlett-Packard, Fort Collins, U.S.A.) and a plotter (HP-7470A, Hewlett Packard, San Diego, U.S.A.). A full description of the detector's possibilities is given elsewhere [24, 25]. A LKB programmable fraction collector was used to collect peaks of interest (SuperRac, LKB, Bromma, Sweden).

HPLC system II consisted of a Waters (Milford, U.S.A.) 6000A pump, a Rheodyne Model 7125 injection valve with a pre-column instead of the sample loop, and a Kontron (Zürich, Switzerland) Uvikon 725 UV detector. A two-channel BD-9 recorder (Kipp & Zonen, Delft, The Netherlands) was connected directly to the detector. A HP-3390A integrator (Hewlett-Packard, Avondale, U.S.A.) was used to measure peak height or peak area.

HPLC system III was the same as System II except for the detector. A Bioanalytical System (BAS, West Lafayette, U.S.A.) Model LC4B/17 electrochemical detector was used in system III. The electrode was glassy carbon with an Ag—AgCl electrode as reference. The applied potential was in all cases 1.1 V.

Mobile phases

Mobile phase I: A: 2 ml/l *tert.*-butylamine in water (pH adjusted to 7.5), B: 2 ml/l *tert.*-butylamine in acetonitrile. Gradient: from 95%A + 5%B to 55%A + 45%B in 15 min and from 55%A + 45%B to 35%A + 65%B in 45 min (total run time was 60 min). Flow-rate: 1 ml/min.

Mobile phase II: 0.005 *M* 1-heptanesulphonic acid in 0.5% acetic acid (pH

4.3) mixed with methanol (35:65). Nonylamine was added to the mixed mobile phase (1.5‰) and the flow-rate was 1.5 ml/min.

Mobile phase III: Methanol—water (40:60) with a flow-rate of 1.3 ml/min.

Mobile phase IV: 0.2 *M* phosphate buffer (pH 6.5)–methanol (50:50) with a flow-rate of 1.5 ml/min.

Mobile phase V: 0.1 *M* phosphate buffer (pH 6.5)—acetonitrile (50:50) with a flow-rate of 1.5 ml/min.

Pre-columns

The pre-columns were made by modifying a Knauer cartridge column (Knauer, Oberursel, F.R.G.) with the dimensions 30 × 4 mm I.D. The cartridge and one set of endfittings were modified in such a way that it was possible to have a pre-column (6 × 4 mm I.D.) housed in a normal 30-mm cartridge holder (details available on request). The pre-columns were dry-packed with RP-18, RP-8 (LiChroprep, 40—63 μm, Merck, Darmstadt, F.R.G.), C_8, CN or phenyl (Sepralyte, 40 μm, Analytichem International, Harbor City, U.S.A.). The packing was enclosed by either 10-μm or 30-μm sieves (Micro Lab, Aarhus, Denmark). The pre-column replaces the sample loop on the Rheodyne injection valve (Fig. 1) and the column is therefore used in the backflush mode.

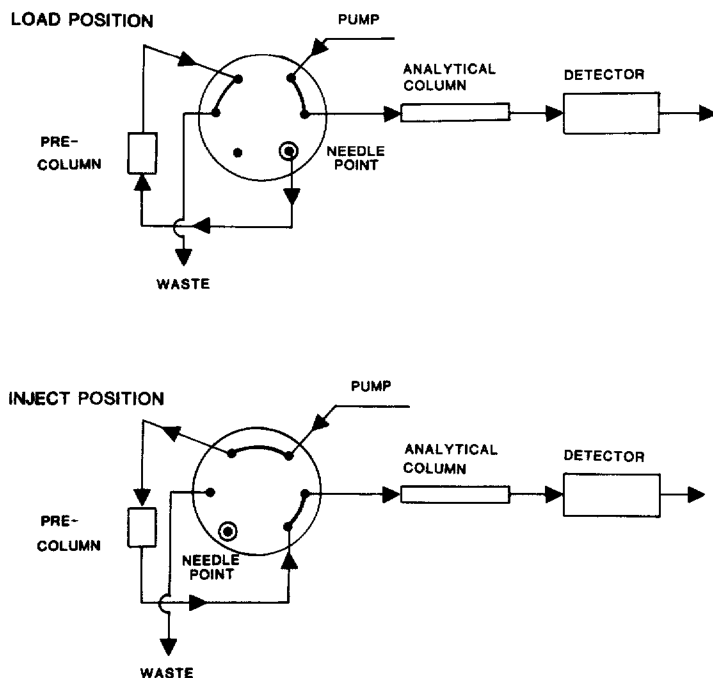


Fig. 1. Flow diagram of the Rheodyne injection valve with the pre-column.

Analytical columns

The analytical columns were all packed in our own laboratory using a Micromeritics stirred-slurry column packer (Model 705, Micromeritics, Norcross, U.S.A.). The pump used was a Stansted pump with a maximum pressure of 518 bar and a flow-rate of 100 ml/min (Stansted Fluid Power, Stansted, U.K.).

The packing material was suspended in 30 ml of methanol—isopropranol (3:7) and placed in an ultrasonic bath for 5 min. After placing the suspension in the reservoir, the columns were packed by pumping methanol into the reservoir. A pressure of 414 bar was used. The columns were all Knauer cartridge columns with an internal diameter of 4 mm and a length of either 250 mm or 100 mm. We have used LiChrosorb RP-18 (5 μm, Merck) and Spherisorb C_{18}, C_8 and phenyl (5 μm, Phase-Sep, Clwyd, U.K.) as packing material. The analytical columns were protected by a 0.5-μm on-line filter (Rheodyne).

Chemicals

The water used during the development of the method was double-distilled and passed through a C_{18} column (LiChroprep, Merck) before use. Using the electrochemical detector, however, made it clear that there were still some impurities present in the water. As a result, an Elgastat UHQ water purification system (Elga, High Wycombe, U.K.) was used to rinse the water. The water from the system is very clean and has been used for all the analyses for some time now. All other solvents were of HPLC or spectroscopic grade. Other chemicals were of the highest purity available.

RESULTS AND DISCUSSION

Development of the pre-column method

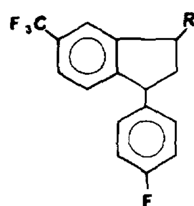
Chromatographic system. The development of the method was performed with HPLC system I, mobile phase I and a column packed with Spherisorb C_8 (5 μm, 250 × 4 mm I.D.). The flow-rate was 1 ml/min and the detector was run at 214, 220 and 229 nm. The detector was programmed to store three UV spectra (200—400 nm) of each peak. The system was used to separate and isolate the metabolites of tefludazine before identification by the microcell NMR technique. Tefludazine is a neuroleptic compound of a new structure synthesized by Lundbeck, Copenhagen, Denmark. Some of the synthesized metabolites possible are shown in Fig. 2.

Pre-columns. The pre-columns were always used in the backflush mode (Fig. 1). We investigated pre-columns with frits or sieves to enclose the packing material. The result was that frits and sieves with fibre-glass filters clogged after a few injections (cf. ref. 11). Consequently, sieves without fibre-glass filters were used.

Sieves with different pore sizes as well as different packing materials have been used. Pore size only had a significant effect on the back-pressure during the injection of a sample if it was < 5 μm. For very large injection volumes (> 30 ml) of solubilized tissue, it was necessary to use 30-μm sieves.

The strong-retaining packing materials (C_{18} and C_8) showed good recoveries and very little peak broadening. The less-strong-retaining materials (CN and phenyl), however, showed larger peak broadening of some of the more polar metabolites. The best results were obtained with a pre-column packed with LiChroprep RP-18 (40—63 μm) and 30-μm sieves.

Because the pre-column is used in the backflush mode, very little peak broadening is seen, even with RP-18 in the pre-column and CN or phenyl in the analytical column. The peak broadening was $< 2\%$ for large (> 20 ml) injections of solubilized tissue samples.



R	NAME
N N-CH_2-CH_2OH	TEFLUDAZINE (1)
N N-CH_2-COOH	Trans-4-(3-(4-fluorphenyl)-6-(trifluormethyl)-1-indanyl)-1-piperazin acetic acid (2)
N NH	1-(3-(p-fluorphenyl)-6-trifluormethyl-1-indanyl) piperazin (3)
-NH-CH_2-CH_2-NH_2	Trans-N-(3-(4-fluorphenyl)-6-trifluormethyl-1-indanyl)-1,2 diaminoethan (4)
-NH_2	3-(4-fluorphenyl)-6-trifluormethyl-1-indan-amin (5)
=O	3-(4-fluorphenyl)-6-trifluormethyl-indan-1-on (6)

Fig. 2. Structure of tefludazine and some of the synthesized metabolites possible.

We also invstigated the effect of flushing the pre-column with different amounts of water after the sample was applied. A sample of 20 ml of solubilized liver, kidney and brain was injected onto the pre-column and flushed with 1, 5, 10, 15, 30 or 60 ml of water. No improvement was seen in the signal-to-noise ratio if 5 or 60 ml of water were used.

Sample preparation

The first injections of serum, spiked with the compounds shown in Fig. 2, were very disappointing because of relatively low recoveries (50—70%). Addition of acetonitrile resulted in recoveries of > 95%. The effect of acetonitrile on the recoveries has been ascribed to a better wettability of the packing by the sample [7, 26]. Psychotropic drugs are, however, known to bind strongly to proteins and another explanation of the effect could be that acetonitrile weakens or destroys these interactions. We investigated the amount of acetonitrile needed and found that 10% was sufficient for serum samples. At this concentration, no precipitation of serum proteins is seen. Analysis of urine samples showed sharp peaks and high recoveries without the addition of acetonitrile. This supports the theory that acetonitrile destroys protein binding.

Osselton and co-workers [27—29] have developed a solubilization technique for tissues using the enzyme subtilisin. Subtilisin is a non-specific proteolytic enzyme that hydrolyses peptide bonds. The enzyme is stable in a wide pH range (7—11) with an optimum temperature of 55°C. Furthermore, the enzyme does not attack conjugates. The solubilization technique that we used was essentially the same as the one described by Osselton [28]. Tissue was homogenized 1:4 in Tris buffer (pH 10), subtilisin A (Novo, Bagsværd, Denmark) was added (1 mg of enzyme to 1 g of tissue) and the sample was incubated at 55°C for 60 min. It was necessary to add acetonitrile to the digested samples to obtain a recovery of > 95%.

Boo and Krohn [7] state that the optimum amount of acetonitrile is 20—30% in samples for direct injection. This corresponds well with our findings, as we found the optimum amount of acetonitrile to be 25%. Werkhoven-Goewie et al. [10] have used the enzyme to destroy the proteins in serum samples in order to obtain better recoveries and to avoid clogging of the pre-column. We found that adding acetonitrile directly to the serum samples resulted in the same recoveries (98%) as with the subtilisin-treated serum. We had no problems with clogged pre-columns after a few injections, as long as

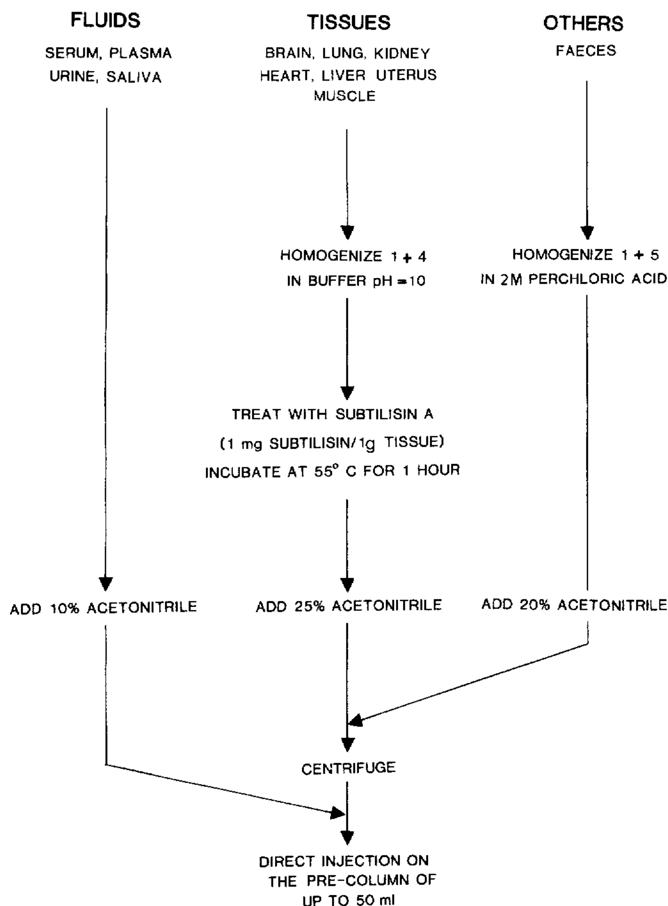


Fig. 3. General work-up procedure.

sieves with a pore diameter larger than 7 μm were used to retain the packing material.

Faeces were homogenized 1:5 in 2 *M* perchloric acid and centrifuged. The amount of acetonitrile needed to give recoveries of > 95% was 20%.

General pre-column procedure

The treatment of all types of samples analysed is shown in Fig. 3.

The pre-column is dry-packed with LiChroprep RP-18 (40—63 μm) and 30-μm sieves. It is flushed with 1 ml of methanol and 1 ml of water. One ml of water is injected, followed by a sample of up to 50 ml (see Fig. 3). The pre-column is flushed with 1 ml of water if the injected sample volume is < 1.5 ml, otherwise 5 ml is used. The sample is injected into the HPLC system by back-flushing it with the mobile phase. The pre-column remains in this position until analysis is complete.

Column life

The number of injections on a pre-column varied, depending on the injection volume. The total volume injected before the column had to be changed, either because of increasing back-pressure or declining sensitivity, was 100—150 ml. As the pre-columns are easy to pack (dry packing), the columns are not regenerated.

The life-time of the analytical columns was the same as if loop injection of extracts had been used. The protecting 0.5-μm on-line filter, however, depending on the type of samples, had to be changed 2—6 times during the life-time (2—4 months) of the analytical column.

EXAMPLES

Metabolite work

All the analyses were performed with LiChroprep C_{18} (40—63 μm) and 30-μm sieves in the pre-column. The HPLC system consisted of HPLC system I, mobile phase I and a column packed with Spherisorb C_8 (5 μm, 250 × 4 mm I.D.).

After an injection of 5 ml of tissues, serum or faeces taken from undosed rats and treated as described in Fig. 3, the resulting chromatograms showed no interfering peaks.

The effect of the addition of acetonitrile to the samples is seen in Fig. 4, where the resulting chromatograms from analysis of a liver spiked with 80 μg of the standards (Fig. 2) are shown. All samples, except urine, showed the same improvement after the addition of acetonitrile.

A chromatogram resulting from analysis of pre-treated faeces spiked with 100 μg of the standards (Fig. 2) is shown in Fig. 5.

We have used this method for metabolite separation and isolation for about 2 years now. One example is given in Fig. 6, where the chromatogram from an injection of 40 ml of solubilized liver from a dosed rat is shown.

HPLC system I, mobile phase II and a column packed with Spherisorb phenyl (5 μm, 250 × 4 mm I.D.) were used to isolate an unknown metabolite of irindalone (see Fig. 7). The peak was collected after a 14-ml injection of

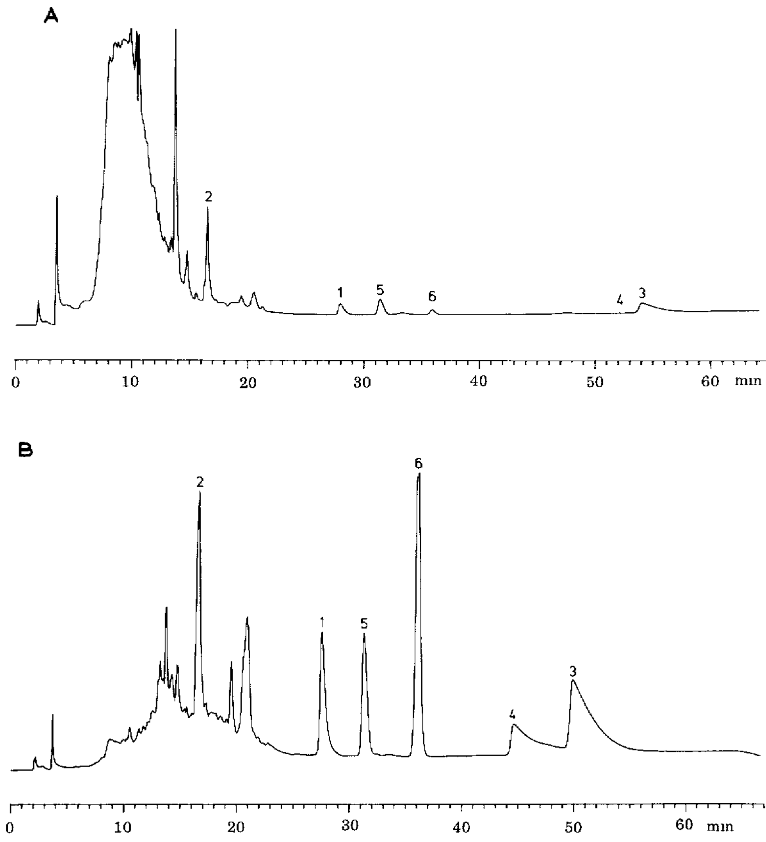


Fig. 4. (A) Chromatogram obtained after injection of 4 ml of digested liver spiked with 80 μg of the standards (no addition of acetonitrile) (B) Same as A, but after addition of 25% acetonitrile. Numbers refer to Fig. 2. HPLC conditions: HPLC system I, mobile phase I and a column packed with Spherisorb C_8 (5 μm, 250 × 4 mm I D.).

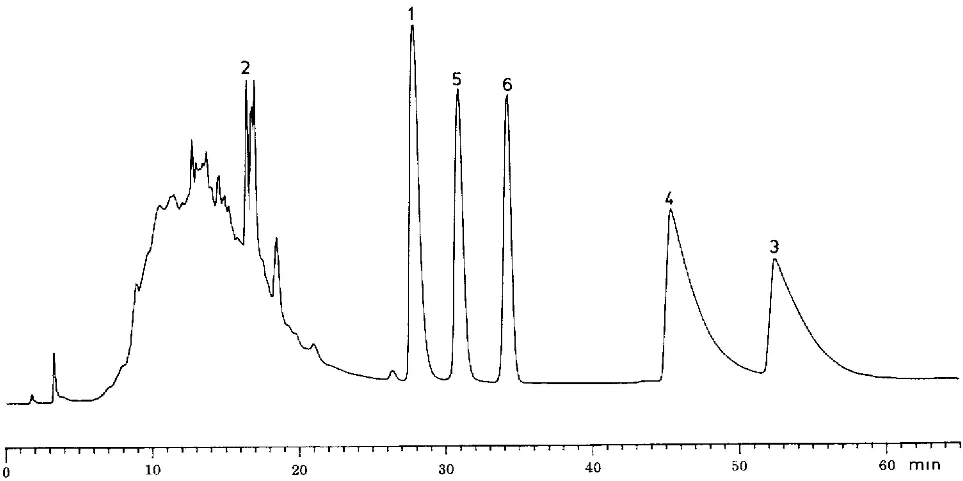


Fig. 5. Chromatogram of 4.5 ml of faeces spiked with 100 μg of the standards. Numbers refer to Fig. 2. HPLC conditions: see Fig. 4.

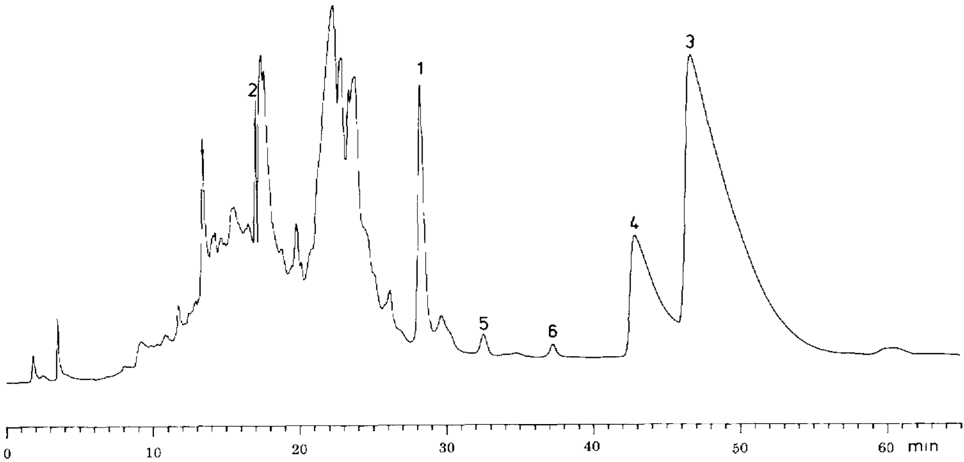


Fig. 6. Chromatogram of 40 ml of solubilized liver from a dosed rat. Numbers refer to Fig. 2. HPLC conditions: see Fig. 4.

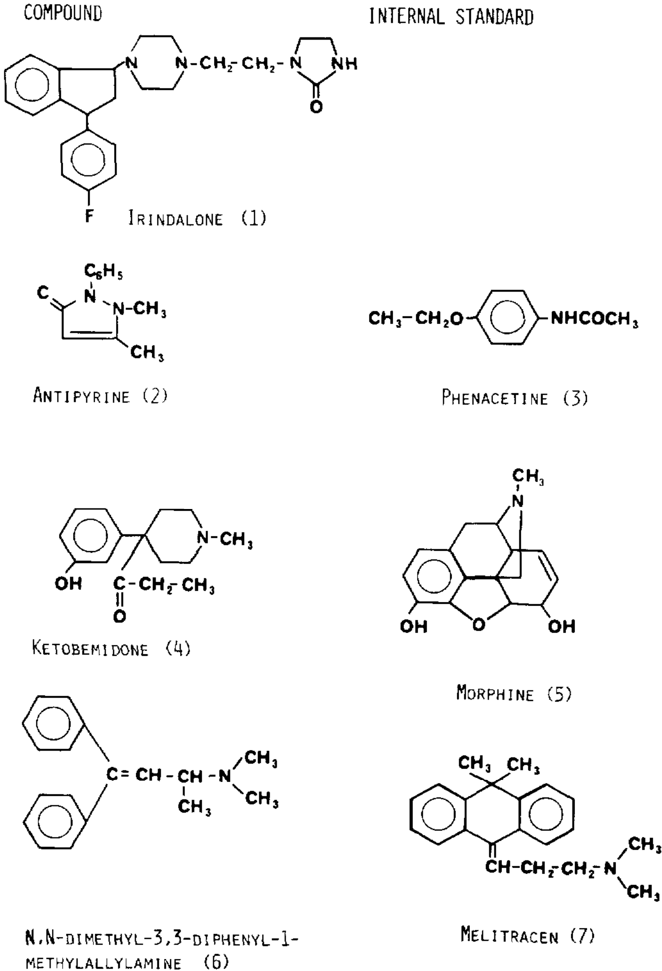


Fig. 7. Structure of some of the compounds analysed.

serum from a dosed dog. Using the microcell NMR technique, it was possible to show that the most likely structure of the metabolite was the N-oxide.

The metabolic work shows that it is possible to use the pre-column technique to clean up large volumes of biological samples. The pre-column packed with C_{18} material can be used as a standard column, as all the metabolites will be retained on it.

Routine analysis

All the analyses were performed with a pre-column packed with LiChroprep C_{18} (40—63 μm) and 30-μm sieves.

The technique has also been used for routine analysis for one year (4000 samples). We have found the within-day precision for these analyses to be better than 2% and the day-to-day precision to be better than 3.5%. The temperature of the analytical column in these analyses was maintained at 40° C by a column oven (Micro Lab).

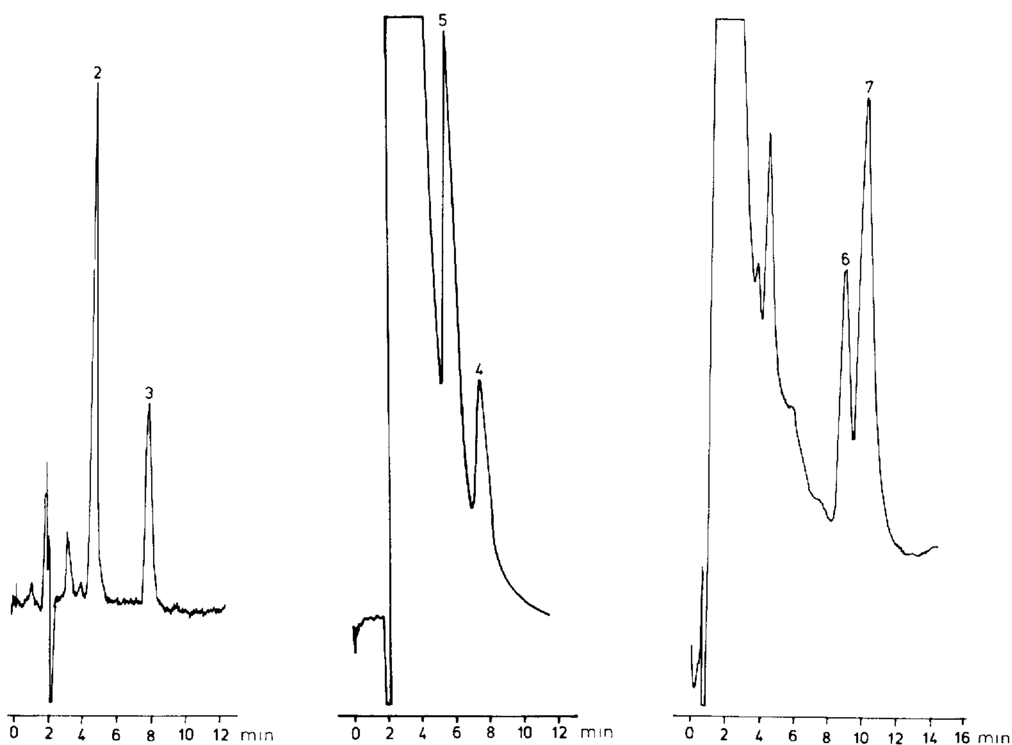


Fig. 8. Chromatogram from analysis of antipyrine in 10 μl of saliva from a rat. Numbers refer to Fig. 7. HPLC conditions: HPLC system II, mobile phase III and a column packed with LiChrosorb RP-18 (5 μm, 250 × 4 mm I.D.).

Fig. 9. Chromatogram from analysis of ketobemidone in 1 ml of serum. Numbers refer to Fig. 7. HPLC conditions: HPLC system III, mobile phase IV and a column packed with Spherisorb C_{18} (5 μm, 250 × 4 mm I.D.).

Fig. 10. Chromatogram from the analysis of N,N-dimethyl-3,3-diphenyl-1-methylallylamine in 1 ml of serum. Numbers refer to Fig. 7. HPLC conditions: HPLC system III, mobile phase V and a column packed with Spherisorb phenyl (5 μm, 100 × 4 mm I.D).

The structures of the analysed compounds are shown in Fig. 7.

Antipyrine clearance is widely used for the quantitative assessment of microsomal liver function [30, 31]. HPLC system II, mobile phase III and a column packed with LiChrosorb RP-18 (5 μm, 250 × 4 mm I.D.) has been used by routine to detect antipyrine in saliva from rats (recovery 100%, detection limit 0.1 ng/ml). Fig. 8 shows a chromatogram from this analysis after injection of 10 μl of saliva.

Ketobemidone (Fig. 7) is a relatively polar narcotic analgesic, which we have analysed using the pre-column technique. HPLC system III, mobile phase IV and a column packed with Spherisorb C_{18} (5 μm, 250 × 4 mm I.D.) were used for the analysis (recovery 95%, detection limit 1 ng/ml). Fig. 9 shows a chromatogram after injection of 1 ml of serum.

A spasmolytic substance (N,N-dimethyl-3,3-diphenyl-1-methylallylamine, Fig. 7) has also been analysed using HPLC system III, mobile phase V and a column packed with Spherisorb phenyl (5 μm, 100 × 4 mm I.D.) (recovery 97%, detection limit 4 ng/ml). A chromatogram obtained after the injection of 1 ml of serum containing this lipophilic compound is shown in Fig. 10.

A comparison of Figs. 9 and 10 shows that it is possible to analyse a wide polarity range with this one material. Furthermore, it is possible to have strong-retaining pre-columns, ensuring high recovery and less-strong-retaining materials in the analytical column without appreciable bond broadening.

The pre-columns used have long life-times (100—150 ml of injected volume), which most likely can be ascribed to two factors. Firstly, the use of packing material with 40—63-μm particles and the use of sieves with 30-μm pores. Secondly, to the addition of acetonitrile to the samples before they are injected onto the pre-column.

CONCLUSION

The pre-column technique with solubilization is a very powerful technique for work-up of all types of biological samples. The pre-column technique has several advantages over the extraction technique: (i) loss due to binding to glassware is avoided, (ii) the recovery is near 100%, (iii) it is simple, (iv) it is fast, (v) it is cheap, and (vi) very large volumes can be injected, resulting in enrichment factors of ca. 200. The treatment of the tissue samples with subtilisin A is a very mild treatment as no degradation of the compounds, e.g. the relatively unstable N-oxide of tefludazine, was seen after incubation with the enzyme.

ACKNOWLEDGEMENTS

The technical assistance by Elsebeth Johansen and Lotte Lindgreen as well as the secretarial assistance by Ruth Sparre Andersson are gratefully acknowledged.

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