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A SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC PRE-COLUMN TECHNIQUE FOR INVESTIGATION OF DRUG METABOLISM IN BIOLOGICAL FLUIDS*

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SUMMARY

A simple reversed-phase high-performance liquid chromatographic pre-column technique for investigation of drug metabolism is described. This rapid method circumvents the "classical" sample purification via extraction by direct purification and enrichment of the sample on the pre-column. Almost 100% recovery of a drug (aminopyrine) and its metabolites from biological fluids is achieved. This is in strong contrast to "classical" sample preparation which allows a recovery of 30–100% depending on the polarity of the investigated compound. The procedure described has been successfully applied to the investigation of the metabolic pattern of aminopyrine in rat plasma and cell incubation media.

INTRODUCTION

For separation and isolation of drugs from biological fluids, high-performance liquid chromatography (HPLC) is often the method of choice because this technique can be applied to compounds of greatly different polarities as is often the case when comparing a drug with its metabolites[1–3]. Furthermore, semipreparative HPLC is one of the most efficient and rapid methods of isolating metabolites in amounts suitable for structure elucidation and pharmacological tests. A drug and its metabolites are often separable by reversed-phase HPLC, however, proteins (*e.g.*, from plasma, liquor or cell incubation media) and inorganic compounds have to be removed in time-consuming work-up procedures, which are the most inaccurate steps owing to the often unknown partition coefficients for all tested compounds. If these by-products are not removed, the lifetime of the column is dramatically reduced and overloading of the packing material with constituents of biological fluids causes a loss in column efficiency. In biological samples interesting compounds are often present at

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low concentrations and may be accompanied by excessive amounts of high-molecular-weight material (*e.g.*, proteins) or salts. Therefore, both enrichment of investigated compounds and sample clean-up has to be carried out prior to analysis.

Pre-columns have been used for enrichment procedures[4–7] as well as for sample clean-up[8–11]. Recently a fully automated method was described for analysis of drugs in biological fluids using pre-columns for simultaneous sample clean-up and enrichment[12]. Unfortunately, this method requires expensive equipment such as an autosampler and time-controlled pneumatic column switching valves. Such a modified HPLC system is especially suitable for routine analysis.

In this communication a pre-column technique is described, which is accessible for manual injection, replacing the sample loop of a manual syringe-loading injector by a pre-column, filled with reversed-phase material. As mentioned above, "classical" sample pretreatment procedures often suffer from low recovery especially for polar compounds. Using the present method a mixture of a drug and its metabolites can be isolated and separated from biological fluids with almost 100% recovery.

EXPERIMENTAL

Apparatus

All separations were performed with a 1010B liquid chromatograph (Hewlett-Packard, Böblingen, G.F.R.) modified with a syringe-loading sample injector (Model 7125; Rheodyne, Berkely, CA, U.S.A.) at 40°C. The pre-column (50 × 4.6 mm I.D.; Dr. Knauer, West Berlin, G.F.R.) was dry filled with LiChroprep RP-8 (particle size 25–40 μm; E. Merck, Darmstadt, G.F.R.) and replaces the sample loop in the injection valve (see Fig. 1). The analytical column was prefilled with LiChrosorb RP-18 (particle size 5 μm, 250 × 4 mm I.D.; E. Merck). Detection was performed with a variable-wavelength detector (SpectroMonitor II; Laboratory Data Control, Riviera Beach, FL, U.S.A.) at 257 nm.

Reagents

Aminopyrine (DMAAP) and its metabolites MAAP, AAP, AcAAP and FAAP (see Table I) were supplied by Hoechst (Frankfurt/M, G.F.R.). All reagents were of research grade, except acetonitrile which was of chromatographic grade (Li-

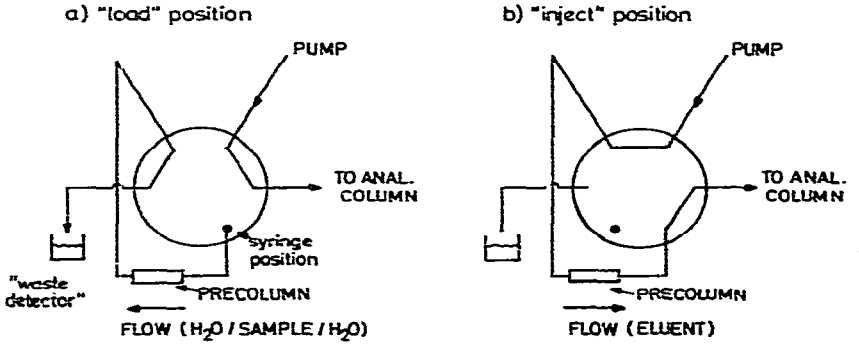
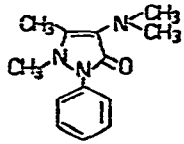
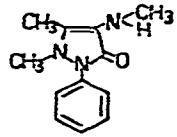
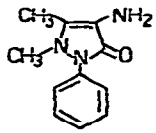
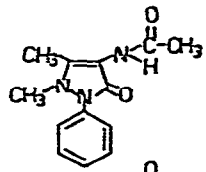
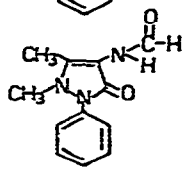
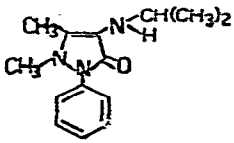


Fig. 1. Schematic flow diagram of the manual pre-column purification and enrichment system.

TABLE I

STRUCTURES AND ABBREVIATIONS OF AMINOPYRINE, SOME MAJOR METABOLITES AND THE INTERNAL STANDARD

Formula	*Abbreviation*	*Name*
	DMAAP	1-Aminopyrine (4-dimethylaminoantipyrine)
	MAAP	Methylaminoantipyrine
	AAP	Aminoantipyrine
	AcAAP	Acetylaminoantipyrine
	FAAP	Formylaminoantipyrine
	i-pAAP	Isopropylaminoantipyrine (internal standard)

Chrosolv, E. Merck) and water which was triply distilled before use. Recovery from serum was carried out with standardized serum (Biotest, Frankfurt/M, G.F.R.).

Pre-column purification and concentration

The pre-column was purged in the "load" position of the injection valve with 1 ml of water (see Fig. 1a). Then, depending on the desired enrichment factor, 50–1000 µl of sample were loaded on the pre-column and highly hydrophilic substances (*e.g.*,

salts and proteins) were washed out with another ml of water. The waste line leads to a beaker containing water–acetonitrile (50:50), to detect the proteins ("waste detector").

The sample was injected by backflushing it with the chromatographic eluent to the analytical column (see Fig. 1b), and the pre-column remains in the "inject" position until analysis is completed.

Extraction

To compare the results of pre-column sample preparation with "conventional" prepared samples, 2 ml of serum spiked with aminopyrine, its metabolites MAAP, AAP, AcAAP, FAAP and i-pAAP as internal standard were diluted with 1 ml of water, and extracted three times with 1 ml of dichloromethane–2-propanol (95:5). The extracts were dried with anhydrous sodium sulphate, filtered and the sodium sulphate was washed twice with 1.5 ml dichloromethane–2-propanol solution. The combined extracts were evaporated to dryness (rotary evaporator), the residue was dissolved in 200 μl of water and an aliquot of 50 μl was injected for analysis.

Preparation of isolated hepatocytes

The preparation technique is mainly in accordance with the method of Berry and Friend[13] as modified by Siess *et al.*[14]. Viability was checked by trypan blue exclusion 30 min after incubation.

RESULTS AND DISCUSSION

The metabolism of aminopyrine has been the subject of extensive studies (*e.g.*, refs. 15–26), however the problem of quantitative recovery of the metabolites is still not solved. Because the metabolism of drugs in isolated hepatocytes is often highly correlated with "*in vivo*" metabolism[27–29], an HPLC method was developed for separation and quantification of aminopyrine and its metabolites in biological fluids, *i.e.*, blood plasma and cell incubation media.

Aminopyrine and its major metabolites are separable from aqueous standard solutions by reversed-phase gradient elution. Therefore, we applied the pre-column technique to detect these substances in plasma and cell incubation media. Sample concentration and purification are carried out simultaneously on the pre-column. It still remains to determine whether the preconcentration is affected by polar contaminants (like salts or proteins). Drugs are also often bound to serum proteins and therefor the serum protein binding could influence their adsorption in the reversed-phase material.

The recovery of aminopyrine and its major metabolites was determined from aqueous standard solutions first, using the pre-column for concentration of the sample prior to analysis. The results are collected in Table II and the values demonstrate that the recovery of all substances is almost 100%.

The serum was spiked with the identical compounds as in the preceding experiment and due to higher background interference only a slightly lower correlation coefficient is observed. Fig. 2 shows a typical chromatogram and Fig. 4 the calibration curve for DMAAP and FAAP recovery from serum which is in the region of 100% (*cf.*, Table II). This demonstrates unequivocally that binding to the non-polar

TABLE II

REGRESSION DATA FOR THE RECOVERY OF AMINOPYRINE AND ITS METABOLITES (n = 4)

Substance	*Slope*	*Intercept*	*Correlation coefficient*
(a) From water (using pre-column sample concentration)			
DMAAP	0.9502	0.2193	0.9999
MAAP	0.9753	−0.0326	0.9995
AAP	0.9897	−0.0781	0.9988
AcAAP	0.9784	−0.0769	0.9999
FAAP	0.9176	0.7148	0.9997
(b) From spiked serum (using pre-column sample purification and concentration)			
DMAAP	0.9737	0.0813	0.9999
MAAP	0.9747	−0.2151	0.9999
AAP	0.9725	0.2608	0.9947
AcAAP	0.9872	−0.1605	0.9986
FAAP	0.9501	0.4183	0.9995
(c) From spiked serum (using "classical" extraction and direct injection)			
DMAAP	0.9865	0.4423	0.9993
MAAP	0.9962	−0.3843	0.9999
AAP	0.9763	−0.2582	0.9999
AcAAP	0.4022	0.3384	0.9952
FAAP	0.3280	0.5587	0.9969

reversed-phase material, even for the most polar compounds, is stronger than binding to the serum proteins.

One-day reproducibility of pre-column serum measurements is better than 5% in the examined concentration range (DMAAP, 1.45 ± 0.71%; MAAP, 2.55 ±

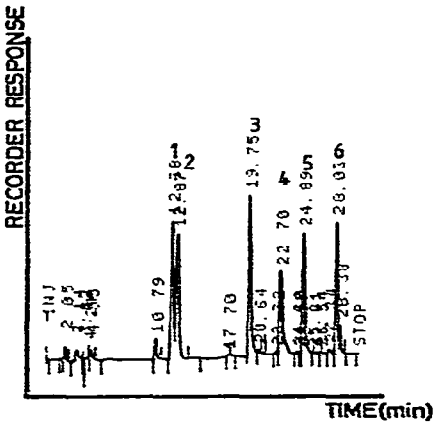


Fig. 2. Typical chromatogram of a spiked control serum sample with one-step sample purification and pre-concentration on the pre-column. Mobile phase: A, 20 mM sodium dihydrogenphosphate in water (pH 8.0, NaOH); B, acetonitrile; gradient from 0 to 8 min isocratic at 13% B, 8 to 30 min from 13 to 43% B (linear). Peaks: 1 = FAAP, 2.58 mg/l; 2 = AcAAP, 2.30 mg/l; 3 = AAP, 2.24 mg/l; 4 = MAAP, 2.16 mg/l; 5 = DMAAP, 2.22 mg/l; 6 = i-pAAP (internal standard). 1 ml water/400 µl sample/2 ml water injected subsequently on the pre-column.

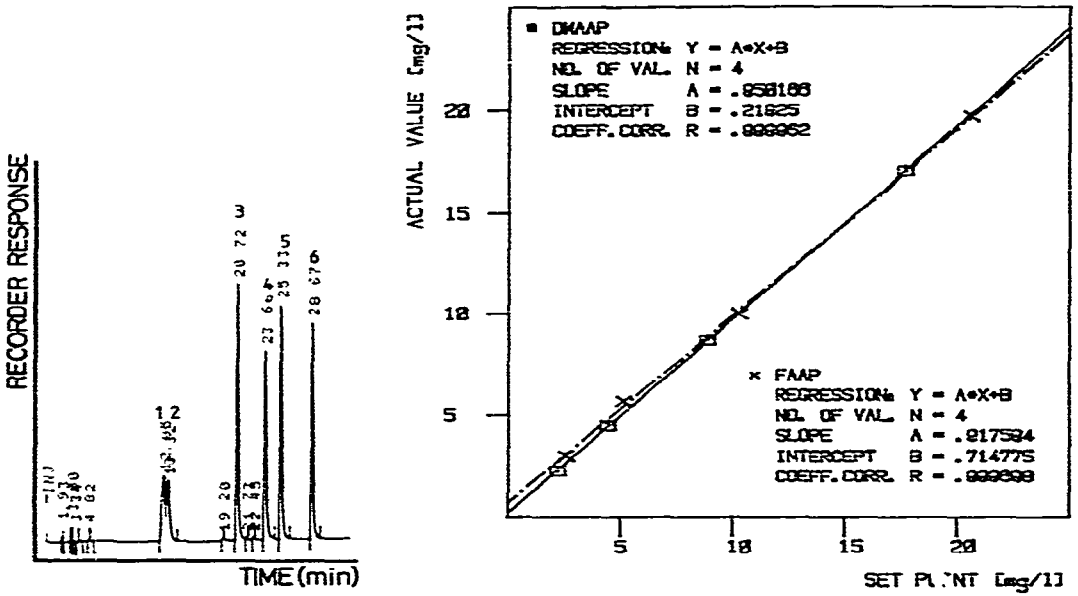


Fig. 3. Analysis of a spiked serum sample with "classical" work-up procedure and analysis by direct injection of 50 µl of extract. For experimental conditions and peak identification see Fig. 2.

Fig. 4. Calibration curves for the recovery of DMAAP and FAAP from spiked serum with pre-column sample purification and enrichment. The regression data for other investigated compounds are collected in Table II.

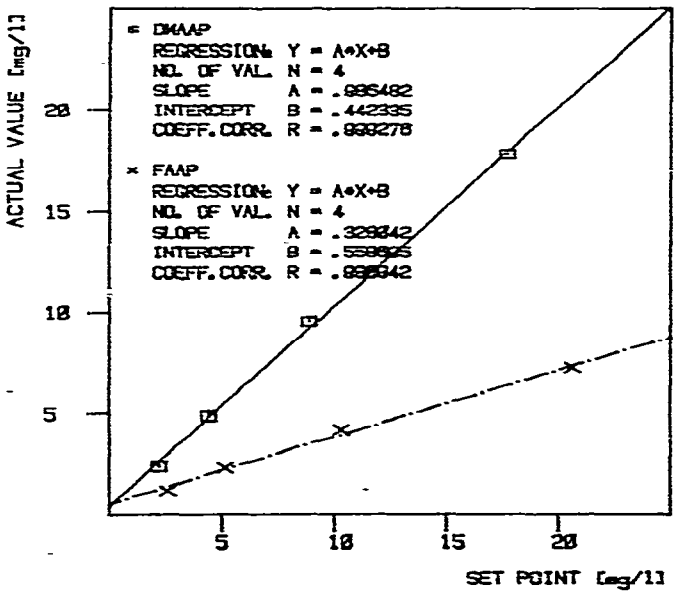


Fig. 5. Calibration curves for the recovery of DMAAP and FAAP of spiked serum obtained by "classical" sample work-up and enrichment. For regression data of other investigated compounds see Table II.

2.01%; AAP, 2.48 ± 2.49%; AcAAP, 1.38 ± 1.51%; FAAP, 2.01 ± 1.37%; $n = 12$ for each compound). Recoveries obtained with different pre-columns show no significant differences (day-to-day reproducibility). If "classical" sample preparation via extraction is used, a remarkable loss of the more polar metabolites occurs (Figs. 3 and 5) due to the change in partition coefficients. Therefore the recovery values for a drug and its metabolites obtained by the "classical" sample pretreatment procedure are often doubtful.

Because pre-column sample pre-treatment is confined to injection steps (*cf.*, water/sample/water) which are carried out during equilibration of the analytical column, no time is wasted on sample preparation. Using the classical procedure, however, the time for sample pre-treatment is as long as the analysis time in this case (about 30 min).

Consequently we used this one-step sample purification and enrichment procedure for the determination of the metabolic pattern of aminopyrine in rat plasma and isolated hepatocytes from rats and mice. Figs. 6 and 7 show typical chromatograms which demonstrate that reversed-phase HPLC combined with pre-column sample work-up is a powerful method for solving complex problems in biotransformation.

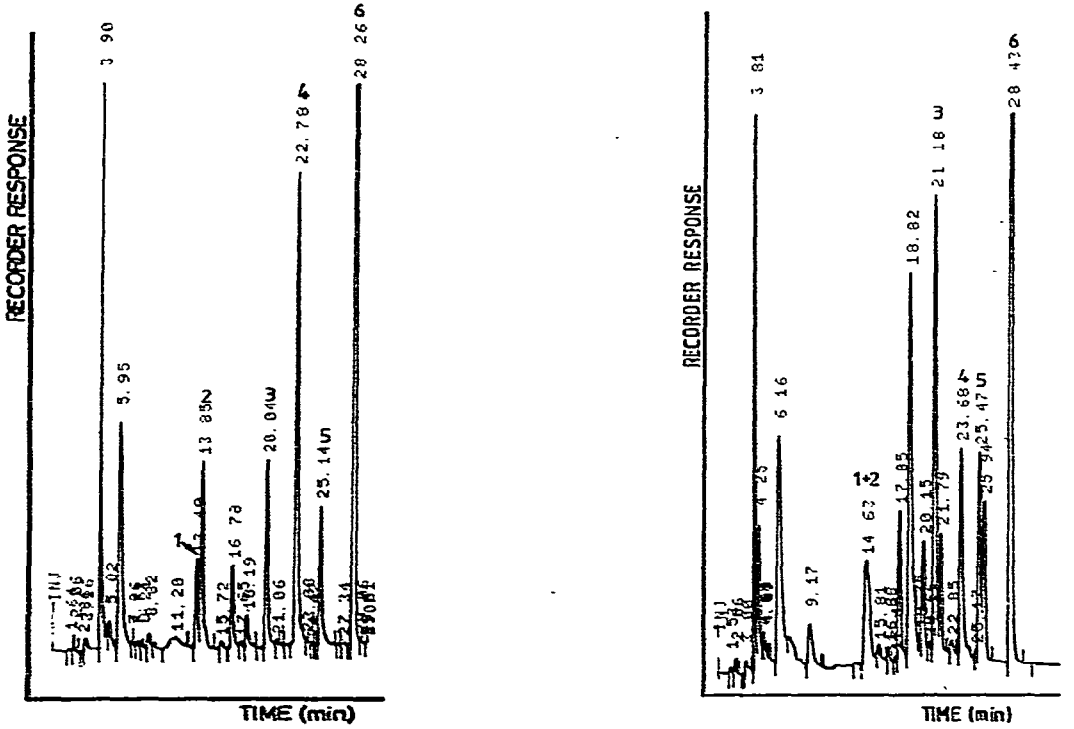


Fig. 6. Analysis of a rat plasma sample 2 h after oral administration of 20 mg/kg with the pre-column technique. Injection volume: 600 μl. For experimental conditions and peak identification see Fig. 2.

Fig. 7. Analysis of the supernatant of a 0.1 mM aminopyrine incubation of isolated rat hepatocytes after 40 min. For experimental conditions and peak identification see Fig. 2. 800 μl were purified and concentrated on the pre-column.

The difficulties of "classical" sample preparation can be easily circumvented by rapid modification of the chromatograph (replacement of the sample loop by the pre-column) and application of the pre-column sample pre-treatment method. The method is especially suitable for the analysis of drugs and metabolites in biological fluids as high recovery is obtained over a wide range of polarity.

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