BIOMEDICAL CHROMATOGRAPHY, VOL. 5, 256–261 (1991)

# Study of the Solid Phase Extraction of Pentoxifylline and its Major Metabolite as a Basis of their Rapid Low Concentration Gas Chromatographic Determination in Serum

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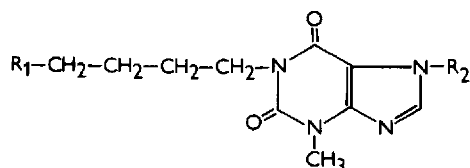
**A gas–liquid chromatographic method for the determination of pentoxifylline and its secondary alcohol metabolite in serum has been developed. The method is based on the combination of solid phase extraction, capillary column separation and nitrogen–phosphorus detection of the analytes. Optimization of the solid phase extraction conditions permitted a low concentration determination, with limits of determination of 2 ng/mL and 10 ng/mL for pentoxifylline and its metabolite, respectively. The simplicity and rapidity of the extraction step was preserved.**

## INTRODUCTION

Pentoxifylline 1-(5-oxohexyl)-3,7-dimethylxanthine (PETX; **1**), is a xanthine derivative acting as a haemorheologic agent (Smith *et al.*, 1986) which is used in the treatment of cerebrovascular and peripheral vascular diseases (Dettelbach and Aviado, 1985). The drug is extensively eliminated ($t_{1/2}$ 0.4–0.8 h; Smith *et al.*, 1986) and one of its major circulating metabolites in plasma is a secondary alcohol metabolite 1-(5-hydroxyhexyl)-3,7-dimethylxanthine (OH-PETX; **2**).




| $R_1$ | $R_2$ | | |
|---|---|---|---|
| $CH_3$–C=O | $CH_3$ | PETX | (1) |
| $CH_3$–CH–OH | $CH_3$ | OH-PETX | (2) |
| $CH_3$–C=O | $CH_3$–$CH_2$–$CH_2$ | PROF | (3) |

Several bioanalytical methods have been developed for the determination of PETX in different physiological fluids, including thin layer chromatography (TLC) (Bauerová *et al.*, 1991), high performance liquid chromatography (HPLC) (Von Stetten *et al.*, 1985; Luke and Rocci, 1986; Garnier-Moiroux *et al.*, 1987; Grasella and Rocci, 1987; Musch *et al.*, 1991) and gas–liquid chromatography (GLC) (Burrows and Jolley, 1985; Burrows, 1987). The limit of determination was in the range of nanograms per millilitre in GLC methods and approximately one order higher in HPLC and TLC. In addition to the determination of the parent drug, the secondary alcohol metabolite was determined in the majority of studies reported.

In the first step of PETX determination, i.e., the transformation of biological samples into analytical ones, liquid–liquid extraction has been used as the main extraction method with dichloromethane as the favoured extraction liquid. Another type of sample clean-up, based on solid phase extraction, was used in three different methods. Rieck and Platt (1984), in a somewhat complicated method, combined the properties of two polar supports (Extrelut and Sep-Pak) to extract PETX and one of its metabolites from human plasma. Von Stetten *et al.* (1985), by using a column switching technique, lowered the detection limit of the drug to about one half in comparison with the majority of HPLC methods. Musch *et al.* (1991) purified and concentrated plasma samples by solid phase extraction using a cyanopropyl sorbent according to a general strategy described by the authors earlier (Musch and Massart, 1988). The solid phase extraction method was also used in combination with TLC. Bauerová *et al.* (1991) extracted PETX and its metabolites on $C_{18}$ silica sorbents before their separation on high performance thin layer chromatographic (HPTLC) plates with a preconcentration zone.

The aim of this work was to combine the advantages of capillary gas–liquid chromatography with nitrogen-selective detection, i.e., sensitivity of the method, with the advantages of solid-phase extraction, i.e., easy manipulation and time saving. A wide-frame study was performed to specify the optimal conditions for solid phase extraction of PETX and its hydroxy metabolite (OH-PETX), with selectivity and simplicity of sample clean-up considered to be the major criteria.

## EXPERIMENTAL

**Instrumentation.** A Hewlett-Packard Model 5880A gas chromatograph was used in conjunction with a Model 7673A Hewlett-Packard autosampler. The chromatograph was equipped with a thermionic selective nitrogen detector (NPD) and a direct injection port. A wide-bore fused silica capillary column HP 1 (30 m × 0.53 mm, film thickness

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0269–3879/91/060256–06 $05.00
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*Received 2 November 1990*
*Accepted 22 November 1990*

2.65 μm; Hewlett-Packard, Vienna, Austria) was used. The GC separations were achieved under temperature programmed conditions using the following sequence: initial temperature 200 °C for 0.01 min; program at 10 °C/min to 250 °C and hold 7 min. The temperature of the injection port and the detector was 300 °C. Nitrogen used as carrier gas was maintained at 25 mL/min. No auxiliary gas was used. The purge activation time was 0.5 min.

**Materials.** PETX, its 5-hydroxy metabolite (OH-PETX) and propentofylline (PROF; 1-(5-oxohexyl)-3-methyl-7-propylxanthine; **3**), used as an internal standard, were synthesized at the Institute for Drug Research, Modra, Czechoslovakia.

Separcol SI C18 3 mL extraction columns (lot 384) (Polymer Institute, Slovak Academy of Sciences, Bratislava, Czechoslovakia) and Supelclean LC18 (lots 0191 and 0236), LC8 (lots 0164 and 0144)) and LCN (lot 0216) 3 mL extraction columns and LC18 (lot 0192) 1 mL extraction columns (Supelco, Bellefonte, USA) were studied in the extraction step.

Analytical grade methanol, acetone, ethyl acetate and dichloromethane were obtained from Lachema, Brno, Czechoslovakia. Acetonitrile and trifluoroacetic anhydride (TFAA) (both puriss, p.a.) were from Fluka, Buchs, Switzerland.

All glassware was cleansed in hydrochloric acid, then silanized with a 5% solution of Surfasil (Pierce, Oud Beijerland, The Netherlands) in benzene.

Human serum used for spiked samples was from the Department of Haematology and Transfusiology, School of Medicine, Comenius University, Bratislava, Czechoslovakia.

**Evaluation of organic solvents for solid phase extraction.** Elution profiles obtained after elution of PETX and OH-PETX from the cartridges tested were used, as this approach was found to be advantageous for the evaluation of different solvents (and also different cartridges) for solid phase extraction (Marko *et al.*, 1990a, 1990b). Separcol SI C18 cartridges were prepared before extraction by flushing with 2 mL of methanol and 1 mL of water. Then 2 mL serum spiked with 2 μg PETX and the same amount of OH-PETX was applied. After passage of the sample solution through the cartridge 3 mL water was applied. The residual water was displaced from the cartridge by under low nitgrogen pressure. For the displacement of retained PETX and OH-PETX, three 1 mL portions followed by one 2 mL portion of methanol, acetonitrile, acetone or ethyl acetate were applied. Individual portions of eluate were collected into 3 mL cone vials (Reacti-Vials, Pierce, Oud-Beijerland, The Netherlands) containing per 2 μg PROF. The solvent was evaporated to dryness at 55 °C under nitrogen. To the dry residue 10 μL TFAA was added to derivatize OH-PETX. Derivatisation took 5 min at 55 °C . After evaporation of the residual TFAA 100 μL dichloromethane was added and the vials were agitated on a Vortex for 10 s. The solution was placed into autosampler vials and 3 μL of the solution was injected into the gas chromatograph. All evaluations were carried out in triplicate.

**Evaluation of cartridges for solid phase extraction.** The same procedure as above, i.e., the procedure utilizing elution profiles, was also applied to the evaluation of the ability of different solid phase cartridges to retain and elute PETX and OH-PETX. Supelclean LC18 (3 mL and 1 mL), LC8 (3 mL), LCN (3 mL) and Separcol SI C18 (3 mL) cartridges were used and the elutions were performed with acetonitrile. In the evaluation of the Supelclean LC18 1 mL cartridges, 1 mL serum was used.

For the evaluation of the selectivity of the $C_{18}$, $C_8$ and CN sorbents towards endogenous compounds from serum, 2 mL of blank serum were applied to the conditioned cartridges. The cartridges were washed with 3 mL water, residual water was displaced under low nitrogen pressure and the endogens retained in the cartridges were eluted with 3 mL acetonitrile. The eluate was evaporated, derivatized and analysed as described in the preceding section.

**Calibration curves.** Various amounts of PETX and OH-PETX (2 ng to 2 μg) and 200 ng of PROF were added to 2 mL human serum. The Separcol SI C18 cartridges were activated by the procedure described, and the samples were applied. The cartridges were then washed with 3 mL water and the residual solvent was displaced under low nitrogen pressure. The analytes were eluted with 3 mL acetonitrile. Acetonitrile was collected into 3-mL cone vials, evaporated and the analytes derivatized and analysed as described in the section on 'Evaluation of organic solvents for solid phase extraction'. The PETX/PROF and OH-PETX/PROF peak area ratios were used to calculate the calibration curves.

**Determination of unknown samples.** Samples of 2–4 mL serum were divided into two parts and placed in 4-mL screwtop vials. A methanolic solution of PROF (50 μg/mL) was added to give a final concentration of the internal standard of 100 ng/mL. The samples were agitated on a vortex mixer for 5 s and carried through the procedure described under 'Calibration curves'.

**Intra-assay precision and accuracy.** Five concentrations of PETX and OH-PETX (1, 2, 10, 100 and 1000 ng/mL), six samples each, were assayed according to the described procedure. The data from this experiment were summarized for each concentration to give the amounts found. The precision was determined on the basis of the obtained coefficients of variation. The accuracy was calculated as follows:

$$(\text{amount found} - \text{amount added})/\text{amount added} \times 100$$

**Inter-assay precision and accuracy.** Analyses of five concentrations of PETX and OH-PETX (1, 2, 10, 100 and 1000 ng/mL) were performed on the first, second, third, sixth and seventh day after their preparation. The data from this experiment were treated in the same way as the intra-assay data.

## RESULTS AND DISCUSSION

### Solid phase extraction

In our previous papers concerning the utilization of solid phase extraction, basic drugs were extracted and subsequently determined by GLC (Marko *et al.*, 1985, 1986; Marko, 1987, 1988, 1989). A considerable increase in selectivity was achieved by including a special washing step with an eluent which could not elute the basic drugs determined. This type of solid-phase extraction, referred to as selective solid-phase extraction (SSPE), was theoretically discussed in our most recent papers (Marko *et al.*, 1990a, 1990c). As a

result of the high selectivity of SSPE, low concentration determinations could be achieved.

Basic drugs interact with modified silica sorbents by hydrophobic interactions and also by polar interactions with acidic sites of the silica surface (Unger and Lork, 1988; Marko *et al.*, 1990a). These multimodal interactions can be used to advantage by including an eluent (e.g., acetonitrile) which removes material bound only by hydrophobic interactions, thus cleansing the sorbent without elution of basic analytes. These latter are subsequently eluted with an eluent (e.g., methanol) which disrupts the polar solute–sorbent interactions.

This approach cannot be used in solid phase extraction of neutral drugs since these do not penetrate deeply into the silica surface but have their most favourable interactions near the centre of solvated chains (Unger and Lork, 1988). Therefore the main type of interaction is hydrophobic, which can be broken by a great number of organic liquids (Fig. 1).

Two facts can be seen from Fig. 1: (i) the $C_{18}$ sorbent retains the majority of the solutes applied and therefore the total recovery of solid phase extraction of both PETX and OH-PETX reaches 90%; (ii) elution of the analytes retained on the sorbent does not change substantially with the elution liquid used. The second finding is also valid for ethyl acetate, regardless of the water immiscibility of the liquid. Since the ability of the organic liquids to elute endogenous compounds from serum was also comparable and the backgrounds after serum extractions were very similar for all the liquids tested, the characteristic that determined the choice of the elution liquid for solid phase extraction of PETX and OH-PETX from serum was the evaporation ability of the eluate, which proved to be optimal for acetonitrile.

Whilst the comparison of various elution liquids did not present great differences, the comparison of various stationary phases exhibited different behavioural patterns. Figure 2 shows elution profiles after elutions of PETX and OH-PETX from 3 mL cartridges packed by $C_{18}$-, $C_8$- and CN-modified silica. In both analytes, phase-to-phase variations were observed. All three phases differ in retention as well as elution ability towards the analytes. The differences were greater in the first characteristic. The best recoveries after elution of PETX and OH-PETX with 5 mL of acetonitrile were obtained with the $C_{18}$ phase (practically 100%); the

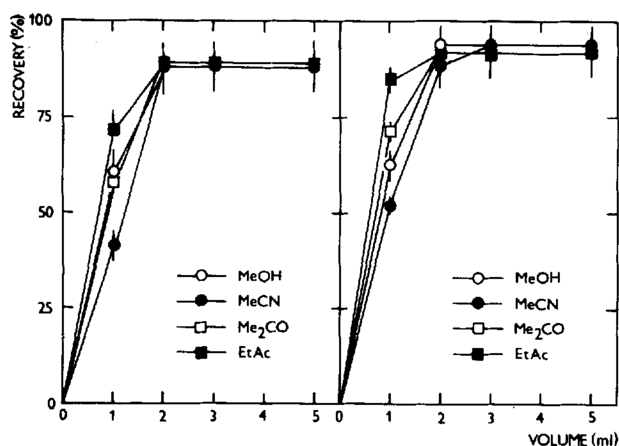


**Figure 1.** Elutions of PETX (left) and its hydroxymetabolite (right) from $C_{18}$ sorbent (Separcol SI C18 cartridges) using different elution liquids.

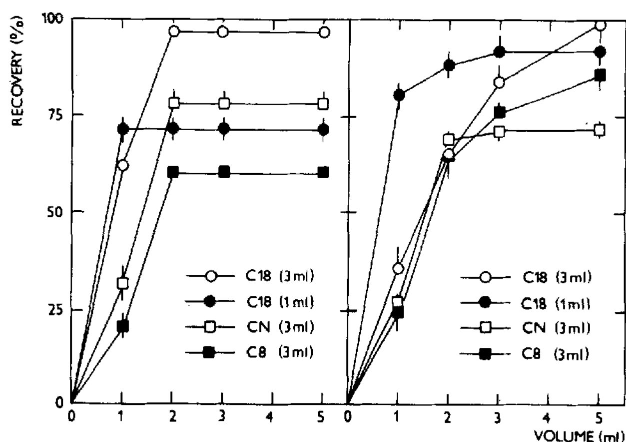


**Figure 2.** Elutions of PETX (left) and its hydroxymetabolite (right) with acetonitrile from differently modified silica sorbents (Supelclean LC cartridges).

other phases displayed lower retention abilities (Table 1). Less than 70% recovery of the $C_8$ phase in the PETX extraction and of the CN phase in the OH-PETX extraction made the use of these two phases for simultaneous solid phase extraction of both compounds questionable.

Lot-to-lot variations were not observed. In both types of sorbents, i.e., in the LC18 and LC8 cartridges, comparative elution profiles of two lots were practically identical for PETX and for OH-PETX.

On the other hand, there was a difference between elution profiles of PETX and OH-PETX using $C_{18}$ cartridges with a different amount of sorbent (Fig. 2). Cartridges with the lower amount of sorbent showed, as expected, a steeper rise of elution curves; however, recovery of analytes was lower in comparison with the 3 mL cartridges—in the case of PETX too low to use 1 mL cartridges for practical purposes (Table 1).

Another important property of sorbents for solid phase extraction is their relative ability to extract endogenous compounds from physiological fluids and thus to influence the signal-to-noise ratio of subsequent analytical methods. In the solid phase extraction of neutral drugs, the only way to influence the selectivity is usually in the choice of extraction sorbent. The chromatograms in Fig. 3 show "noise" after solid phase extraction and subsequent GC determination of extracts from human serum using differently modified silicas. The backgrounds are displayed using two levels of attenuation. Approximate heights of signals of PETX and OH-PETX (10 ng/mL for each) are also displayed (dashed lines). As evident from Fig. 3, no difference is observable between the $C_{18}$ and the $C_8$ phase, while the cyanopropyl-modified silica retains endogenous compounds to such an extent that determination of low concentrations of the analytes is practi-

**Table 1. Recovery of PETX and OH-PETX from serum, using differently modified silica sorbents**

| Sorbent | Recovery ± SD (%)[a] PETX | OH-PETX |
|---|---|---|
| $C_{18}$ (3 mL) | 97 ± 1 | 99 ± 9 |
| $C_{18}$ (1 mL) | 72 ± 3 | 92 ± 7 |
| $C_8$ (3 mL) | 60 ± 2 | 87 ± 2 |
| CN (3 mL) | 78 ± 3 | 71 ± 2 |

[a]SD = standard deviation of three experiments.

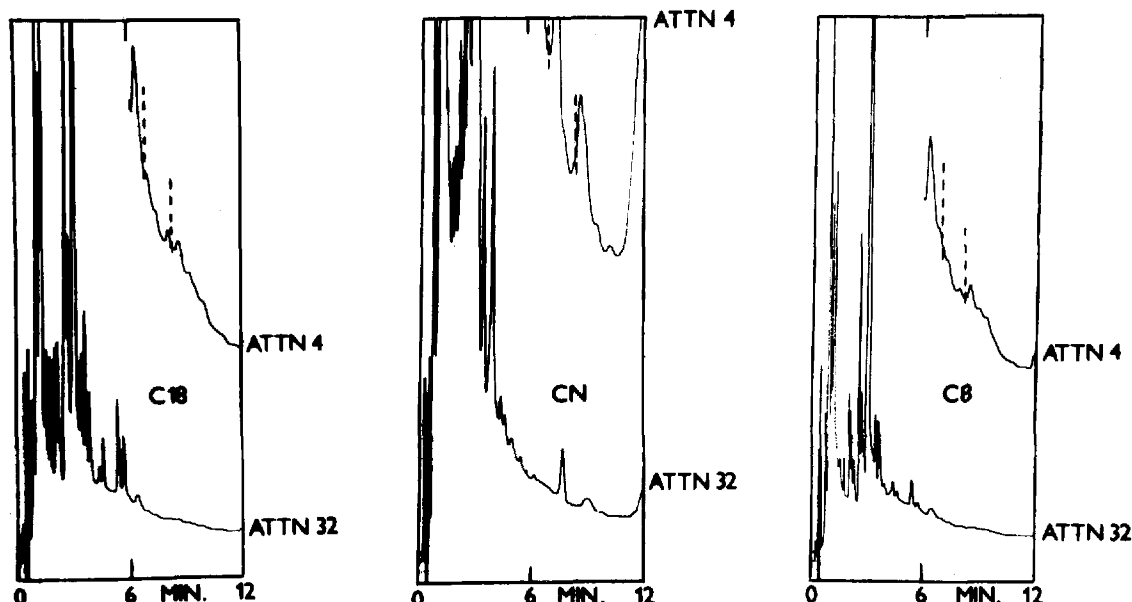


**Figure 3.** Chromatograms of blank serum extracts after solid phase extraction with differently modified silica sorbents (Supelclean LC cartridges).

cally impossible. This low selectivity of the CN phase discriminates against this sorbent more than does the low recovery of PETX and OH-PETX, showed in the previous experiment.

Manufacturer-to-manufacturer variation, frequently discussed in connection with solid phase extraction (McDowall, 1989) was not confirmed in our experiments. Comparison of $C_{18}$ sorbents of two manufacturers, i.e., Separcol SI C18 cartridges and Supelclean LC18 cartridges, did not yield any substantial differences from the point of view of elution profiles obtained after elution of both PETX and OH-PETX from the cartridges with acetonitrile (Figs. 1 and 2) or from the point of "noise" after solid phase extraction (Figs. 3 and 4). A moderately lower retention of PETX in Separcol SI C18 cartridges in comparison with Supelclean LC18 cartridges is counterbalanced by faster elution of OH-PETX from the first type and thus by the possibility of eluting the analytes totally by 3 mL of the eluent. This manufacturer-to-manufacturer invariation was also observed in our laboratory in the study of solid phase extraction of other types of drugs from serum (Marko, unpublished results). In choosing cartridges it was necessary to assess their secondary properties, i.e., the amount of a residue from the sorbent itself in the extract and the feasibility of evaporation of an eluate from cartridges. In both characteristics, Separcol SI C18 cartridges displayed better properties. After elution of these cartridges, no evaporation residue was observed, while with Supelclean LC18, a white precipitate remained on the bottom of the vials after evaporation. After performing solid phase extraction of PETX and OH-PETX from serum, a considerably shorter evaporation time was recorded after extraction with Separcol SI C18 than with Supelclean LC18 cartridges. This finding can be explained by the greater dead volume of the latter type with a resulting higher retention of water.

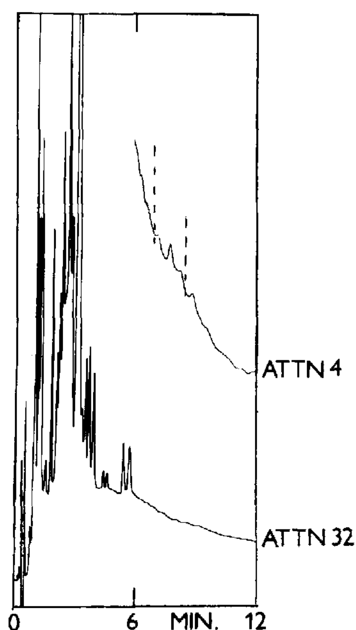


**Figure 4.** Chromatogram of blank serum extract after solid phase extraction with Separcol SI C18 cartridge.

In conclusion, Separcol SI C18 cartridges containing octadecylsilanized silica as a sorbent were chosen as the extraction tool and acetonitrile as the elution liquid.

### Gas–liquid chromatography

Figure 5 shows three chromatograms. The first is after solid phase extraction of blank canine serum, the second is of serum spiked with PETX, OH-PETX and PROF, and the third was obtained after solid phase extraction and subsequent GC determination of a real sample. Peaks of PETX, OH-PETX and PROF are baseline-separated with retention times of 8.4, 6.8 and 9.6 min, respectively. No interferences from endogenous compounds were observed. In the chromatogram of the real sample more peaks appeared than in the spiked sample. Metabolites other than OH-PETX are the most probable source of these peaks. A search for structures of the potential metabolites by means of GLC/MS is in progress.

The calibration curves of PETX and OH-PETX were constructed from eight points each and situated within

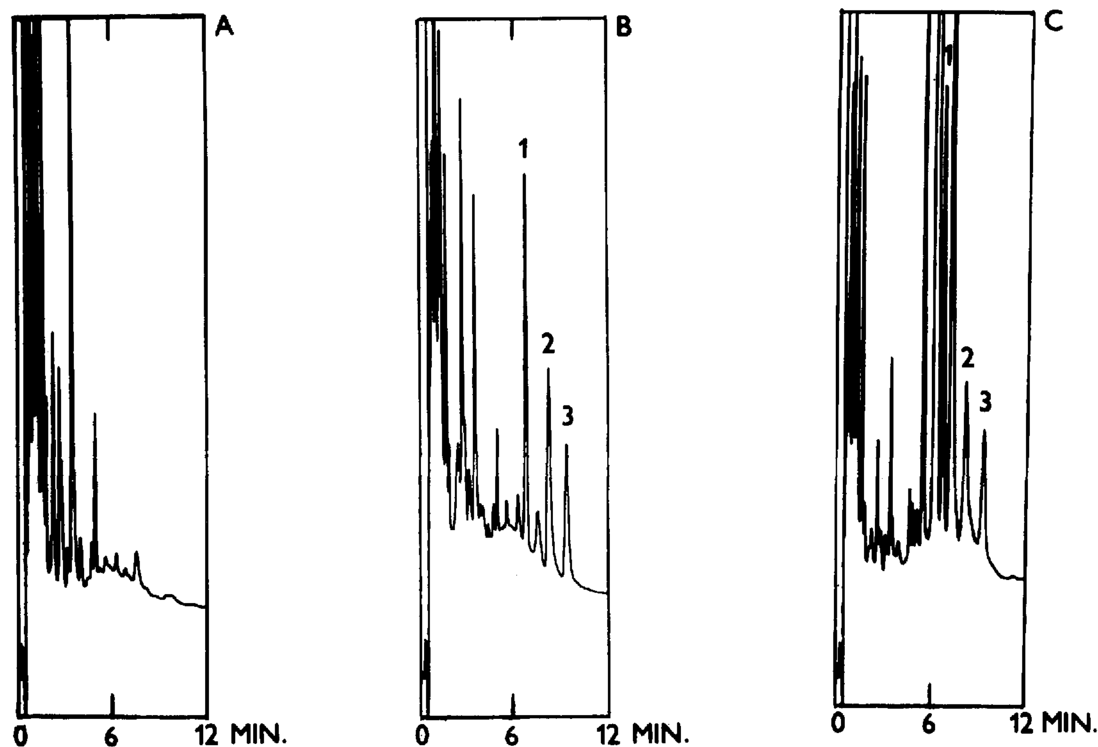


**Figure 5.** Chromatograms of serum extracts: (A) serum free from drugs; (B) serum spiked with PETX (100 ng/mL), OH-PETX (100 ng/mL) and the internal standard (PROF) (100 ng/mL); (C) serum obtained from real experiment (calculated concentrations: 92 ng/mL and 144 ng/mL for PETX and OH-PETX, respectively). Peaks: (1) OH-PETX, (2) PETX, (3) PROF.

**Table 2. Intra-assay accuracy and precision of determination of PETX (six samples each concentration)**

| Concentrations added (ng/mL) | Concentrations determined ± SD (ng/mL) | Accuracy (%) | Precision (%) |
|---|---|---|---|
| 1 | 1.4 ± 0.1 | 40.0 | 10.0 |
| 2 | 2.1 ± 0.3 | 5.0 | 13.7 |
| 10 | 10.1 ± 0.4 | 1.0 | 4.0 |
| 100 | 100.6 ± 4.1 | 0.6 | 4.1 |
| 1000 | 1029.8 ± 46.2 | 3.0 | 4.6 |

**Table 3. Intra-assay accuracy and precision of determination of OH-PETX (six samples each concentration)**

| Concentrations added (ng/mL) | Concentrations determined ± SD (ng/mL) | Accuracy (%) | Precision (%) |
|---|---|---|---|
| 1 | 1.1 ± 0.6 | 10.0 | 53.5 |
| 2 | 1.4 ± 0.3 | 60.0 | 21.5 |
| 10 | 12.2 ± 1.7 | 22.0 | 13.9 |
| 100 | 98.2 ± 1.9 | 1.8 | 1.9 |
| 1000 | 1051.1 ± 66.9 | 5.1 | 6.4 |

**Table 4. Inter-assay accuracy and precision of determination of PETX (five determinations each concentration)**

| Concentrations added (ng/mL) | Concentrations determined ± SD (ng/mL) | Accuracy (%) | Precision (%) |
|---|---|---|---|
| 1 | 1.5 ± 0.2 | 46.0 | 12.7 |
| 2 | 2.2 ± 0.2 | 10.0 | 8.7 |
| 10 | 10.2 ± 0.7 | 2.0 | 6.7 |
| 100 | 100.7 ± 4.6 | 0.7 | 4.6 |
| 1000 | 1047.8 ± 33.8 | 4.8 | 3.3 |

**Table 5. Inter-assay accuracy and precision of determination of OH-PETX (five determinations each concentration)**

| Concentrations added (ng/mL) | Concentrations determined ± SD (ng/mL) | Accuracy (%) | Precision (%) |
|---|---|---|---|
| 1 | 1.0 ± 0.6 | 0.0 | 64.5 |
| 2 | 1.2 ± 0.1 | 40.0 | 9.6 |
| 10 | 10.4 ± 0.8 | 4.0 | 7.6 |
| 100 | 99.9 ± 2.0 | 0.1 | 2.0 |
| 1000 | 1037.3 ± 59.0 | 3.7 | 5.7 |

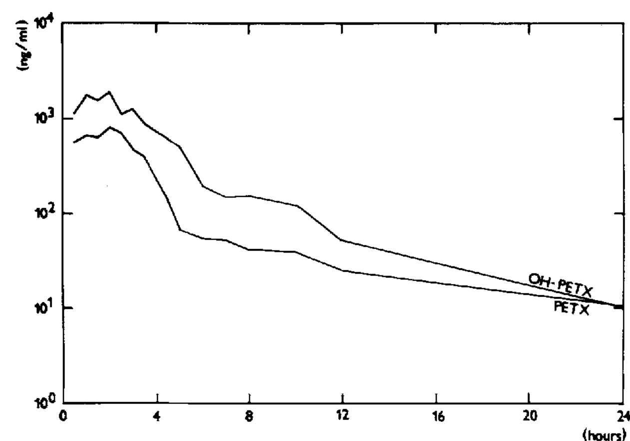


**Figure 6.** Serum concentrations of PETX and OH-PETX after administration of the drug to a dog (Agapurin retard, 400 mg *per os*).

three orders. Linearity was achieved in the whole concentration range studied with coefficients of correlation higher than 0.99 in both instances. Therefore an

internal standard calibration method offered by the manufacturer for the level four terminal of the HP 5880A gas chromatograph could be used (Hewlett-Packard, 1979).

The accuracy and precision data obtained are presented in Tables 2–5. The data show better accuracy and precision for the unchanged drug than for the hydroxy metabolite in practically all the concentrations tested. This enabled us to choose the limit of determination, i.e., the lowest concentration with an acceptable accuracy and precision—for PETX at 2 ng/mL, while for OH-PETX at a five times higher concentration, 10 ng/mL.

A method was developed for the determination of PETX and OH-PETX in bioavailability studies of sustained release drug formulations of PETX. An example of the time dependence of concentrations of PETX and OH-PETX after oral administration of the drug to a dog in a dose of 400 mg is shown in Fig. 6.

## Acknowledgements

The authors thank Supelco Inc., Bellefonte, USA, for material support.

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