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Note

Phenacetin estimation from biological samples by high-performance liquid chromatography

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The estimation of phenacetin, a commonly consumed analgesic, in biological materials has presented problems regarding specificity and sensitivity. Brodie and Axelrod[1] developed a colorimetric method, but this suffers from both deficiencies and in addition is a difficult and time-consuming procedure. Several other spectrophotometric methods have been developed[2,3] and, although more simple, still suffer from the lack of specificity and sensitivity. A gas chromatographic method has been described by Prescott[4]. However, this method requires derivatization, and others[5] have encountered difficulties when using this method. Pantuck *et al.*[6] described a non-derivatization method but this method is limited by the large volume of biological material required.

We have developed a variety of procedures using high-performance liquid chromatography (HPLC) to suit all biological samples. The specificity and sensitivity achieved are excellent and the procedures are relatively simple.

EXPERIMENTAL AND RESULTS

Phenacetin was obtained from Sigma (St. Louis, Mo., U.S.A.) and its purity was verified by multiple melting point determinations, UV–visible spectral analysis, and thin-layer chromatography (TLC)[7]. Further examination by HPLC revealed a single peak.

Phenacetin was administered to pentobarbital-anesthetized dogs in a dose of 90 mg/kg. Blood and urine samples were collected at 15-min intervals and immediately cooled to −5°. On completion of the experiment both kidneys were removed and immediately frozen in acetone and dry ice.

Samples of kidney tissue weighing between 0.2 and 1.5 g were homogenised with a Brinkmann (Westbury, N.Y., U.S.A.) Polytron and diluted to 4 ml with distilled water. Two milliliters of the homogenate were alkalinized to pH 12 with NaOH and extracted with 20 ml of benzene. Fifteen milliliters of benzene were then evaporated using Brinkmann evaporating beakers and the residue was dissolved in 200 μl dioxane. 5–100 μl of this solution were used to run a chromatogram (Fig. 1) using Waters Assoc. M6000 pumps, a UV spectrophotometer with the wavelength set at 254 nm, a Model 660 solvent programmer, and a Model U6K injector. The column

was 61 cm × 2 mm I.D. stainless steel packed with Porasil A. The solvent system was 2,2,4-trimethylpentane–1,4-dioxane (7:3) at a flow-rate of 2 ml/min (Fig. 1A). An alternate solvent system, dioxane–chloroform (1:9), can provide a satisfactory alternative (Fig. 1B). The concentration of dioxane may have to be varied when using solvents from different sources.

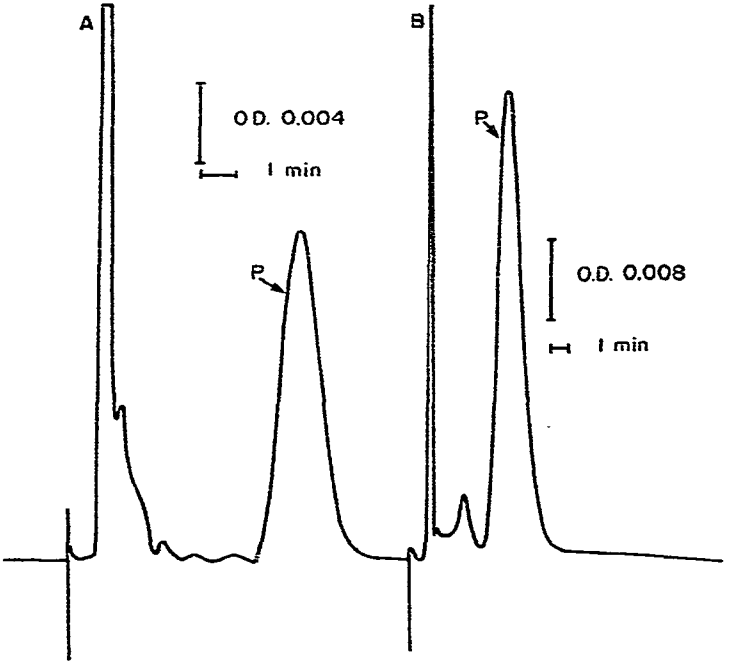

Fig. 1. (A) Chromatogram of tissue extract showing phenacetin (Peak P) using a 61 cm × 2 mm I.D. stainless-steel column packed with Porasil A and with 2,2,4-trimethylpentane–1,4-dioxane (7:3) as the solvent system. (B) Same column as (A) with the solvent system dioxane–chloroform (1:9).

Plasma samples between 0.5 and 2 ml were similarly alkalinized with NaOH and extracted with 20 ml benzene and 15 ml of this extract were evaporated as described above. The residue was then dissolved in 200 μl of methanol and 5–100 μl were used to run a chromatogram (Fig. 2) using the same equipment with a 30 cm × 4 mm I.D. stainless-steel column packed by Waters Assoc. with 10-μ particle size μBondapak-C_{18} (reversed-phase). The solvent was methanol–0.01 *M* NH_4CO_3 (3:7) at a flow-rate of 2 ml/min.

Urine samples were used untreated or filtered through a 0.5-μ Millipore filter. 5–100 μl of urine were chromatographed (Fig. 3) using the same solvent system as used for plasma but in the ratio 2:8 at a flow-rate of 2 ml/min.

Further urine samples were hydrolysed with aryl sulphatase and β-glucuronidase (Sigma Type H 1) in acetate buffer at pH 5. This was then treated in a manner identical to that used for a plasma sample with the addition of an 0.1 *N* HCl wash of the benzene phase following the alkaline extraction, to remove both free amine and

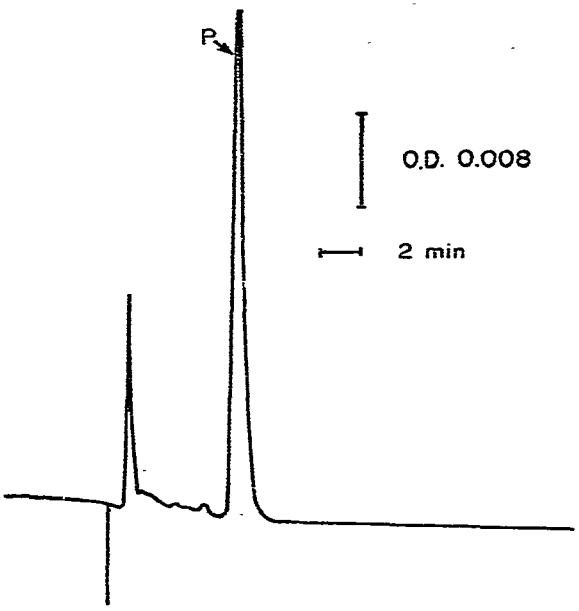

Fig. 2. Chromatogram of plasma extract showing phenacetin (Peak P) using a 30 cm × 4 mm I.D. stainless-steel column packed with μBondapak-C_{18} and with methanol–0.01 *M* NH_4CO_3 (3:7) as the solvent system.

phenolic metabolites of phenacetin[1,7]. Samples were chromatographed on the normal and the reversed-phase columns (Fig. 4). In each system described external standards of phenacetin were treated in a manner identical to that used for their respective biological samples. All samples were injected at a temperature of 5° and the columns were maintained at ambient temperature. Solvents used were Burdick and Jackson UV-grade.

DISCUSSION

Both methods produce satisfactory results. The system using a 61 cm × 2 mm I.D. column packed with Porasil A and with 2,2,4-trimethylpentane–1,4-dioxane (7:3) as the solvent system was selected as an alternative to the reversed-phase μBondapak-C_{18} column, as there was a peak which persistently interfered over a wide variety of solvent compositions in the latter method. The nature of this compound is uncertain, as it was neither an organic acid nor a base.

Hydrolysis of urine samples by β-glucuronidase and aryl sulphatase and its subsequent extraction shows two compounds on TLC[7]. This is also shown in Fig. 4 when chromatographed by HPLC. The compound has not been identified, but it is possibly N-hydroxyphenacetin, as this is the only known metabolite of phenacetin which will partition into benzene from an acid or alkaline aqueous solution. It exists normally in the urine as the conjugate which is insoluble in benzene. This compound also interferes with the spectrophotometric and colorimetric assays[1–3] of urine if any hydrolysis has taken place. It is usually undetectable in plasma and tissue. Reproducibility was

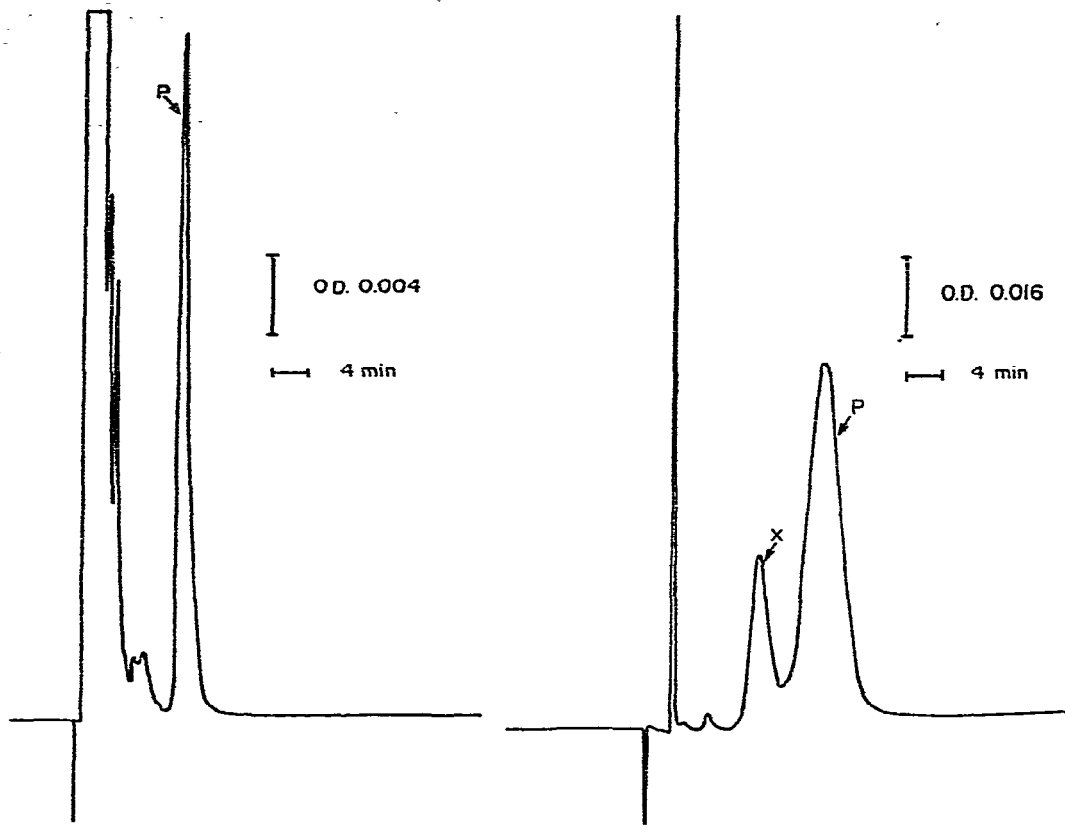

Fig. 3. Chromatogram of urine showing phenacetin (Peak P) using a 30 cm × 4 mm I.D. stainless-steel column packed with μBondapak-C_{18} and with methanol–0.01 *M* NH_4CO_3 (2:8) as the solvent system.

Fig. 4. Chromatogram of enzymatically hydrolysed urine extract. Column identical to that used in Fig. 1. Solvent, chloroform. Peak P is phenacetin; Peak X is unidentified.

determined by fourteen separate injections of a standard sample over an 8-h period while the instrument was in continuous operation. Quantitation was determined by peak height and by area under the curve using perimetry. The standard error was ± 1.2% in both instances.

Detector response was linear over the entire range of concentrations from 0.1–100 μg/ml when determined by area. Calibration curves were hence unnecessary.

Recovery of phenacetin added to biological samples was 99.7% ± 1.5 S.E.M. for urine, 98.8% ± 1.6 S.E.M. for plasma, and 97.2% ± 1.8 S.E.M. for tissue when compared to a similarly treated external standard.

The sensitivities of the above methods are such that approximately 0.1 μg/ml of phenacetin in plasma and urine and 0.5 μg/g of tissue can be readily detected. There are no interfering compounds, as demonstrated by chromatographing thirty samples from dogs not given the drug, and the time taken for each analysis is at most 15 min.

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REFERENCES

1 B. B. Brodie and J. Axelrod, *J. Pharmacol. Exp. Ther.*, 95 (1949) 58.
2 J. E. Wallace, J. D. Biggs, H. E. Hamilton, L. L. Foster and K. Blum, *J. Pharm. Sci.*, 62 (1973) 599.
3 H. L. Gurtoo and B. M. Phillips, *J. Pharm. Sci.*, 62 (1973) 383.
4 L. F. Prescott, *J. Pharm. Pharmacol.*, 23 (1971) 111.
5 B. H. Thomas and B. B. Caldwell, *J. Pharm. Pharmacol.*, 24 (1972) 243.
6 E. J. Pantuck, R. Kuntzman and A. H. Conney, *Science*, 175 (1972) 1248.
7 A. Klutch and M. Bordun, *J. Pharm. Sci.*, 57 (1968) 524.