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Note

# Improved liquid chromatographic analysis of phenytoin and salicylate using radial compression separation

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High-performance liquid chromatography (HPLC) is successfully used for quantitation of the antiepileptic agent phenytoin and the antipyretic—analgesic compound salicylate in biological fluids [1—14]. This paper describes an improved HPLC analytic technique for these two compounds, using a newly developed liquid chromatography separation system. The method is sensitive enough for single-dose pharmacokinetic studies of phenytoin and salicylate in humans or animals, and can easily be modified for therapeutic monitoring.

## EXPERIMENTAL

### *Materials*

Pure samples of phenytoin, tolylbarbital, salicyclic acid, and 3,4-dimethoxybenzoic acid (DMBA) were obtained from Aldrich (Milwaukee, WI, U.S.A.). All other reagents, analytical grade or better, were purchased from commercial sources and used without further purification. Mobile phase components (water, aqueous acetic acid, and acetonitrile) were separately filtered prior to mixing, then degased after mixing.

### *Apparatus and chromatographic conditions*

A Waters Assoc. (Milford, MA, U.S.A.) liquid chromatograph was used. The instrument was equipped with a Model 4000A solvent delivery system, a

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Model 480 variable-wavelength spectrophotometer, and a Model 710B (WISP) automatic sample processor. Detector output (peak height) was quantitated using a Model 730 data module. The separation system was a reversed-phase C-18 radial compression cartridge (spherical 10-μm nonpost-silanized), 10 cm × 5 mm I.D., which was housed in an RCM-100 radial compression module.

For analysis of phenytoin, the mobile phase consisted of water—acetonitrile (70:30). The spectrophotometer was operated at 195 nm, and the mobile phase flow-rate was 0.8 ml/min. For analysis of salicylate, the mobile phase was acetonitrile—water (20:80), to which were added 10 ml of acetic acid per l. The mobile phase flow-rate was 1.0 ml/min, and detector output was quantitated at 305 nm. All analyses were performed at room temperature.

*Stock solutions*

Standard solutions of phenytoin, tolylbarbital, salicyclic acid, and DMBA each were prepared by dissolving 100 mg in 100 ml of pure methanol. Working solutions were prepared by appropriate dilution with methanol. Solutions are stable for at least one year when stored at 4°C.

*Preparation of samples*

For phenytoin analysis, tolylbarbital served as internal standard [4]. A fixed amount (2.5 μg) of tolylbarbital was added to a series of 13-ml round-bottom culture tubes equipped with PTFE-lined screw-top caps. To a series of calibration tubes were then added variable amounts of phenytoin ranging from 0.1 to 10.0 μg. Drug-free control plasma (0.5 ml) was added to each of the calibration tubes; 0.1—1.0 ml of unknown plasma or serum was added to all other tubes. Four ml of benzene—isoamyl alcohol (98.5:1.5) were added to all tubes, and the samples were gently agitated in the upright position on a Vortex mixer for 30—60 sec. After centrifugation at 400 *g*, an aliquot (approximately 3.5 ml) of the organic phase was transferred to a 13-ml tapered glass centrifuge tube. The organic solvent was evaporated to dryness at 40—50°C under mildly reduced pressure. The residue was redissolved in 200 μl of methanol, which was then trasferred to an automatic sampling vial equipped with a limited volume insert. The automatic sampler was programmed to inject 20 μl of each sample.

For analysis of salicylate, DMBA served as internal standard. A constant amount (20 μg) of DMBA was added to a series of tubes. Calibration tubes were prepared by addition of variable amounts of salicylic acid, ranging from 1 to 50 μg, to a series of these tubes. Drug-free control plasma (0.5—1 ml) was added to the calibration tubes, and 0.1—1.0 ml of unknown plasma was added to all other tubes. Samples were acidified by addition of one drop of concentrated phosphoric acid, and then extracted with 4 ml of ethyl acetate—benzene (1:1). The samples were agitated by Vortex mixing, centrifuged, and an aliquot of the organic phase separated to a tapered glass centrifuge tube. The organic phase was evaporated to dryness under mildly reduced pressure, but all samples were placed on a bed of ice to avoid sublimation of salicylate. The residue was redissolved in 200 μl of methanol and prepared for injection by the automatic sampler as described above.

*Clinical studies*

A healthy male volunteer participated in a pharmacokinetic study of intravenous phenytoin after giving informed consent. The subject received a single 300-mg dose of phenytoin sodium (Parke Davis, Ann Arbor, MI, U.S.A.), equivalent to 275 mg of free phenytoin, by infusion into an antecubital vein over a period of 10 min. Multiple venous blood samples were drawn into heparinized tubes during the 72 h after the dose. Concentrations of phenytoin in all samples were determined by the method described above. Using standard pharmacokinetic techniques [15], plasma phenytoin concentrations were used to determine volume of distribution, elimination half-life, and total metabolic clearance.

Another healthy volunteer participated in a two-way crossover study of salicylate pharmacokinetics after giving informed consent. On one occasion, 650 mg of acetylsalicylic acid (aspirin), prepared as the lysine salt (Bayer, Köln, G.F.R.), was infused into an antecubital vein over a period of 5 min. After a washout period of one week, the subject received the same dose of aspirin as two commercially available aspirin tablets (Bayer, New York, NY, U.S.A.) together with 100—200 ml of tap water after an overnight fast. For both studies, multiple venous blood samples were drawn in the 12 h after the dose. Salicylate concentrations in all plasma samples were determined by the method described above. No attempt was made to quantitate the low concentrations of intact aspirin or other metabolites that might have been present.

Using standard kinetic methods [15], salicylate volume of distribution, elimination half-life, and total clearance were determined after the intravenous dose, assuming that the entire 650-mg dose of aspirin was converted to the molar equivalent of salicylate (498 mg). After oral aspirin administration, absolute systemic availability of salicylate was determined by comparison of the total area under the plasma concentration curve with that observed in the same subject after intravenous dosage.

*Experimental study*

An anesthetized adult mongrel dog received a single 500-mg intravenous dose of aspirin as the lysine salt using an experimental preparation described in detail previously [16, 17]. Multiple blood samples were drawn during the 8 h after the dose, and cisteral cerebrospinal (CSF) samples were drawn from an indwelling cannula. Serum and CSF salicylate concentrations were determined as described above.

## RESULTS

*Evaluation of the method*

Under the described chromatographic conditions, phenytoin and tolylbarbital gave two symmetric well-resolved chromatographic peaks (Fig. 1A). Drug-free blank plasma samples were consistently free of endogenous contaminants at the retention times corresponding to the two compounds. The relation of plasma phenytoin concentration to the phenytoin:tolylbarbital peak height ratio was linear to at least 10.0 μg/ml. The equation of a typical

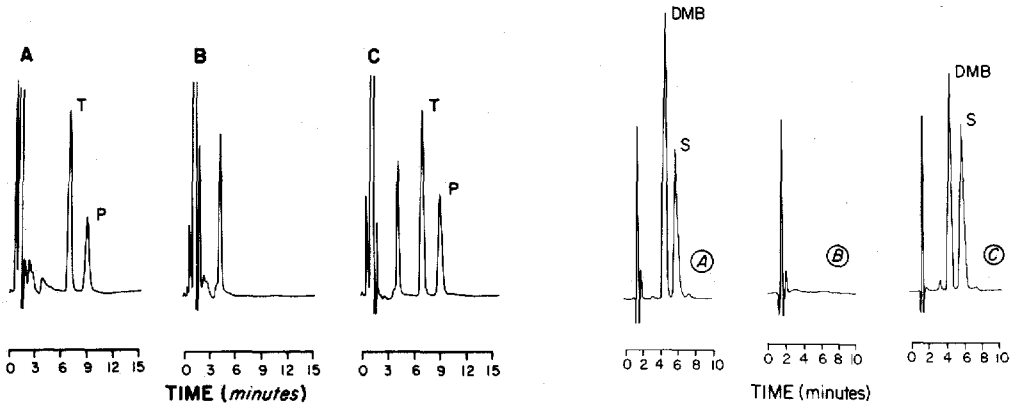


Fig. 1. (A) Chromatogram of a calibration standard containing 1.0 μg/ml of phenytoin (P) and 2.5 μg/ml of tolylbarbital (the internal standard) (T). (B) Chromatogram of a drug-free control plasma extract. (C) Chromatogram of a sample from a subject who had received 300 mg of phenytoin sodium intravenously 12 h previously.

Fig. 2. (A) Chromatogram of a calibration standard containing 10 μg/ml of salicylate (S) and 20 μg/ml of 3,4-dimethoxybenzoate (DMB), the internal standard. (B) Chromatogram of a drug-free control plasma extract. (C) Chromatogram of a sample from a subject who had received a single 650-mg dose of aspirin intravenously 12 h previously.

TABLE I

REPLICABILITY OF IDENTICAL SAMPLES

$n$ = 5 at concentration.

| | Plasma concentration (μg/ml) | Coefficient of variation* (%) |
|---|---|---|
| Phenytoin | 0.1 | 7.2 |
| | 0.25 | 5.0 |
| | 0.5 | 4.0 |
| | 0.75 | 4.6 |
| | 1.0 | 1.7 |
| | 2.5 | 3.1 |
| | 5.0 | 3.6 |
| Salicylate | 2.5 | 1.9 |
| | 5.0 | 3.8 |
| | 10.0 | 9.3 |
| | 25.0 | 3.1 |
| | 50.0 | 5.8 |

*Standard deviation divided by mean.

regression line was $y = 0.373x - 0.031$ ($r = 0.999$), where $y$ is peak height ratio and $x$ is plasma phenytoin concentration. Table I show the coefficients of variation for identical samples at various concentrations. The sensitivity limits are approximately 0.05 μg of phenytoin per ml of original sample.

Salicylate and the internal standard dimethoxybenzoate (DMB) likewise gave two well-resolved symmetric chromatographic peaks (Fig. 2A). No interfering endogenous contaminants were observed. The relation of plasma salicylate concentration to the salicylate:DMB peak height ratio was linear to at least 50 μg/ml. The equation of a typical regression line was: $y = 0.051x - 0.02$ ($r = 0.999$), where $y$ is peak height ratio and $x$ is plasma salicylate concentration. Table I shows the replicability of identical samples at various concentrations. The sensitivity limits are approximately 0.5 μg per ml of sample, and can be extended by minor modifications such as reduction of the amount of internal standard added or increasing the injected volume of the final residue.

*Pharmacokinetic results*

Fig. 3 shows plasma phenytoin concentrations in the volunteer subject. The peak concentration measured immediately after the dose was 24.7 μg/ml. Concentrations then fell in biphasic fashion, with an apparent elimination half-life of 12 h. The apparent volume of distribution was 39 l, and the total metabolic clearance 37 ml/min.

Fig. 4 shows plasma salicylate concentrations after intravenous injection of aspirin. The peak concentration was 55 μg/ml, reached immediately after the dose. Thereafter, concentrations declined biphasically, with an elimination half-life of 1.9 h. The calculated volume of distribution was 10.2 l, and the total metabolic clearance 61 ml/min. After oral aspirin administration, the peak concentration was 33.6 μg/ml, reached at 1.0 h after dosage (Fig. 4). The half-life of elimination following oral administration was essentially identical to that observed after the intravenous dose. Based on comparison

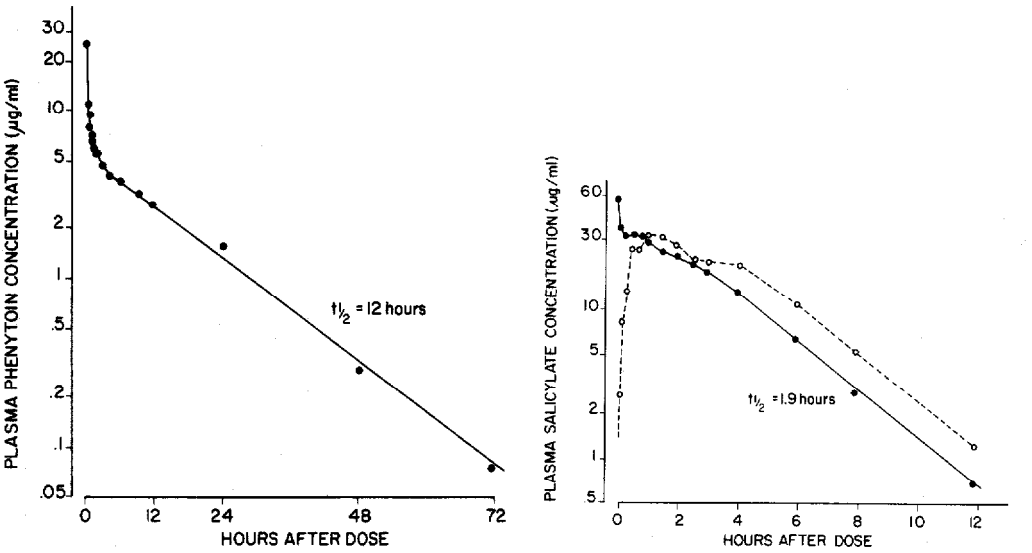


Fig. 3. Plasma phenytoin concentrations in the volunteer subject following a single 300-mg intravenous dose of phenytoin sodium.

Fig. 4. Plasma salicylate concentrations in a volunteer subject after 650-mg intravenous (●——●) and oral (○— — —○) doses of aspirin administered on two occasions.

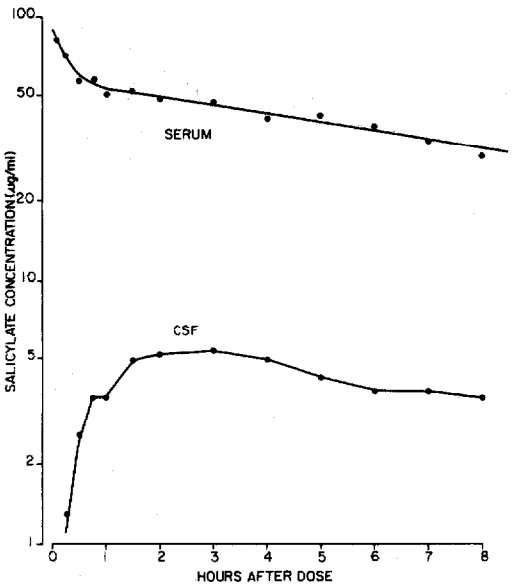


Fig. 5. Serum and CSF salicylate concentrations in the experimental study as described in the text.

of areas under the plasma concentration curve by the two modes of administration, absolute systemic availability of salicylate after oral aspirin was calculated to be 100%.

After intravenous aspirin administration in the experimental study, the peak serum salicylate level was 84 µg/ml, reached immediately after the dose (Fig. 5). Salicylate thereafter disappeared biphasically with a terminal elimination half-life of 8.6 h. Salicylate entered CSF slowly with peak concentrations not reached until 3 h after the intravenous dose. CSF concentrations were considerably lower than those in serum. The slow CSF entry probably reflects the relatively poor lipid solubility of salicylate, with correspondingly slow diffusion across the lipoidal blood brain barrier [18]. The incomplete entry reflects the extensive serum protein binding of salicylate, with only the unbound or free component present in serum being available for diffusion into CSF [16, 17].

## DISCUSSION

This paper describes a rapid and sensitive HPLC method for quantitation of phenytoin and salicylate in biologic fluids. The method has advantages over previously described techniques. The same column can be used for analysis of both compounds, so that changing from one assay to the other requires only a shift of mobile phase composition and absorbance wavelength. Second, the use of the automatic injection system allows analysis of up to 100 samples per 24 h by one person working a standard 8-h day, since sample preparation can be done during the work day while chromatography can proceed overnight. Finally, sensitivity and stability of the assay system are enhanced and cost is reduced by use of the radial compression separation system. The

radial compression module applies pressure uniformly to the outside of a flexible-walled cartridge which contains the actual chromatographic material. The external radially-applied pressure forces the wall of the cartridge to conform to the packing material and prevents channel formation, dead spaces (voids), or shifting of the packing. The radial compression cartridges are considerably less expensive than most other HPLC column systems, have a longer life, and will perform well with low mobile phase flow-rates thereby reducing the cost of solvent consumption.

The approach to quantitation of phenytoin and salicylate utilizes internal standardization with structurally related compounds. Biologic samples are extracted into an organic solvent and chromatographed after evaporation and reconstitution. The sensitivity limits of the method are more than sufficient for essentially any type of clinical or basic pharmacokinetic study of these compounds, as demonstrated by the studies described above. During usual therapeutic use, steady-state plasma concentrations of phenytoin and salicylate are considerably higher than those encountered in single-dose pharmacokinetic studies. Thus the present methodology can easily be adapted to therapeutic monitoring either by using small aliquots of plasma (0.1 ml) or by addition of larger amounts of internal standard with a wider calibration range.

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