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HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC DETERMINATION OF FORPHENICINE IN MOUSE SERUM AND MUSCLE BY PRE-COLUMN FLUORESCENCE DERIVATIZATION USING 1,2-DIAMINO-4,5-ETHYLENEDIOXYBENZENE AS FLUOROGENIC REAGENT

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SUMMARY

A simple and sensitive high-performance liquid chromatographic method has been developed for the determination of forphenicine in biological samples. Forphenicine in the deproteinized sample is converted by reaction with 1,2-diamino-4,5-ethylenedioxybenzene into a fluorescent derivative. The derivative is separated on a reversed-phase column (TSK gel ODS-120T) by isocratic elution with acetonitrile–30 m*M* phosphate buffer (pH 6.5) (5:1, v/v) and monitored fluorimetrically. The method allows the quantification of forphenicine in serum (100 μl) and muscle (0.1 g) of mice dosed with forphenicine or forphenicinol. The limits of detection (signal-to-noise ratio of 3) are 7.35 pmol/ml in serum and 5.36 pmol/g in muscle. The distribution of forphenicine and forphenicinol in the mouse serum and muscle after oral administration of these compounds is also described.

INTRODUCTION

Forphenicine [L-(4-formyl-3-hydroxyphenyl)glycine] is a microbial fermentation product and an inhibitor of alkaline phosphatase [1]. As an analogue of forphenicine, forphenicinol [*S*-2-(3-hydroxy-4-hydroxymethylphenyl)glycine] was also synthesized [2]. Both compounds have effects in experimental animals for enhancement of delayed-type hypersensitivity and for inhibition against murine transplantable tumours [3,4]. From these pharmacological aspects, forphenicine and forphenicinol have been studied as possible therapeutic drugs for cancer, microbial infection and muscular dystrophy [5].

Methods for the determination of both forphenicine and forphenicinol are required not only for pharmacokinetic studies but also for the therapeutic examination of whether the drugs can enter the target organs. No methods for

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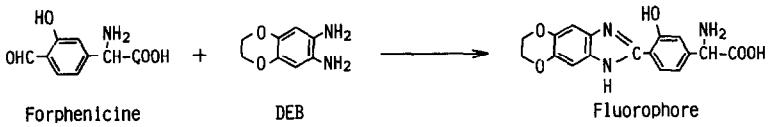

Fig. 1. Fluorescence derivatization of forphenicine with DEB.

monitoring forphenicine in biological samples appear to be available, although a few methods for forphenicinol have been reported [6–8].

Forphenicine is an amino acid analogue having a formyl group and is not a fluorescent compound. We have recently found that reaction with 1,2-diamino-4,5-ethylenedioxybenzene (DEB), which was developed as a fluorogenic reagent for aromatic aldehydes especially having a phenolic hydroxy group [9], can convert forphenicine into a highly fluorescent derivative (Fig. 1).

This paper describes a simple and sensitive method for the quantification of forphenicine in mouse serum and muscle by means of high-performance liquid chromatography (HPLC) with fluorescence detection utilizing the above derivatization. We also report a comparison of forphenicine concentration with the forphenicinol concentration in samples of mouse after oral administration of either forphenicine or forphenicinol. The determination of forphenicinol was performed by a previously reported method [8].

EXPERIMENTAL

Materials and solutions

Deionized, distilled water was used throughout. Forphenicine and forphenicinol were obtained from the Institute of Microbial Chemistry (Tokyo, Japan). DEB dihydrochloride was synthesized according to the method of Parys [10]. All other chemicals were of analytical-reagent grade. Solutions of reagents were prepared in water, unless indicated otherwise.

Administration and sample preparation

Inbred mice (C57BL, five weeks old, weight ca. 18 g) were used. The mouse received a 30 mg/kg oral dose of forphenicine or forphenicinol dissolved in saline by using a conductor needle. After the administration, the mouse was fed only with water until being killed by exanguination from a carotid artery. The blood was collected in a centrifuge tube and serum was separated. Trapezius muscle of the mouse was also quickly collected, washed with saline and weighed after removing the saline with filter-paper.

A 100-μl portion of serum was mixed with 100 μl of 1.5 *M* perchloric acid and 50 μl of water (or standard solutions of forphenicine or forphenicinol for the calibration graph), and the mixture was centrifuged at 1000 *g* for 15 min for deproteinization. The sliced muscle (ca. 0.1 g) was homogenized with 150 μl of 8.4 m*M* acetic acid. To the homogenate, 150 μl of water (or standard solutions of forphenicine or forphenicinol) and 250 μl of 1.5 *M* perchloric acid were added.

The mixture was centrifuged at 1000 *g* for 20 min for deproteinization. Portions of the deproteinized serum and muscle samples were used for derivatization.

Fluorescence derivatization of forphenicine

A 100-μl portion of the sample solution was mixed with 200 μl of 15 m*M* DEB solution (usable for ca. 20 h when stored at room temperature). The mixture was then heated at 60°C for 30 min. A 100-μl portion of the final reaction mixture was used for HPLC assay.

HPLC conditions and apparatus

The HPLC system consisted of a Toyo Soda 803D high-performance liquid chromatograph equipped with a Rheodyne 7125 syringe-loading sample injector valve (100-μl loop) and a Hitachi F-1000 fluorescence spectrophotometer fitted with a 12-μl flow cell. A column (25 cm×0.4 cm I.D.) of TSK gel ODS-120T (particle size 5 μm) (Tosoh, Tokyo, Japan) was used. The mobile phase was 30 m*M* phosphate buffer (pH 6.5)–acetonitrile (5:1, v/v) at a flow-rate of 1.0 ml/min. The column temperature was ambient (24±4°C).

Uncorrected fluorescence excitation and emission spectra of the eluates were measured with a Hitachi MPF-4 spectrofluorimeter in 10×10 mm quartz cells; spectral band widths of 10 nm were employed for both the excitation and emission monochromators.

RESULTS AND DISCUSSION

Fluorescence derivatization

Forphenicine reacted with DEB in acidic pH to give a fluorescent derivative; maximum yield of the derivative could be attained at pH 2 when the effect was examined by using 50 m*M* phosphate buffer (Fig. 2A). In the present derivatization procedure, however, the residual perchloric acid in the supernatant after the deproteinization could serve to form an acidic medium; a decreased concentration of perchloric acid in the range 1.0–5.0 *M* resulted in an increased formation of the fluorescent derivative (Fig. 2B). However, at a concentration lower than 1.5 *M*, the deproteinization was incomplete. Therefore, 1.5 *M* perchloric acid was used in the present procedure. At this concentration, the pH of the derivatization mixture was approximately 1.0.

Maximum yield of the fluorescent derivative was obtained at a DEB concentration higher than 10 m*M*; 15 m*M* DEB was therefore employed.

Higher temperatures allowed forphenicine to form the derivative more rapidly (Fig. 3). At 60°C, a maximum and constant rate of derivative formation was achieved for a reaction time between 30 and 60 min; heating at 60°C for 30 min was adopted in the procedure.

Determination of forphenicine

Fig. 4 shows typical chromatograms obtained with serum and muscle from a mouse dosed with forphenicine. A fluorescent peak for forphenicine was apparently observed at a retention time of 18.8 min. No interfering peaks arose in the

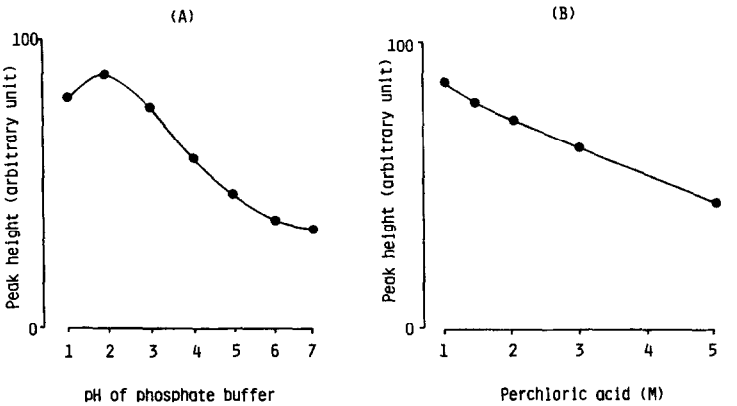

Fig. 2. Effects of (A) pH and (B) the concentration of perchloric acid used for deproteinization on the derivatization of forphenicine. (A) 50 μl each of 10 nmol/ml forphenicine and 50 m*M* phosphate buffer (pH 1.0–7.0) were treated in place of a biological sample solution according to the derivatization procedure; (B) a 50-μl portion of 25 nmol/ml forphenicine was added to 100 μl of a drug-free serum and deproteinized with perchloric acid at various concentrations, and the resulting supernatant was treated according to the procedure.

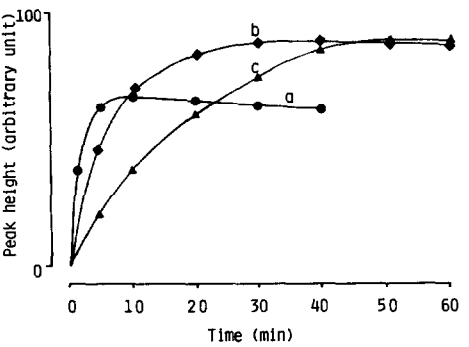

Fig. 3. Effects of reaction time and temperature on the derivatization of forphenicine. A 50-μl portion of 10 nmol/ml forphenicine was added to 100 μl of drug-free serum and then deproteinized, and the supernatant was treated as in the procedure for various periods at temperatures of (a) 100°C; (b) 60°C and (c) 37°C.

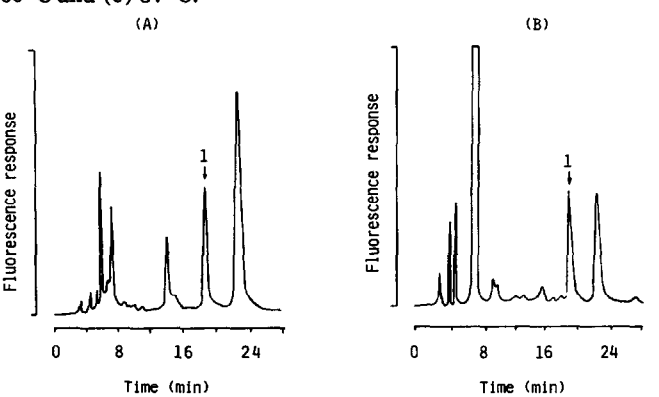

Fig. 4. Chromatograms obtained with (A) serum and (B) muscle 8 h after oral administration of forphenicine to a mouse. Dose: 30 mg/kg. Peaks: 1 = forphenicine; others = endogenous substances in serum or muscle. Concentrations of forphenicine: (A) 1.85 nmol/ml in serum; (B) 1.31 nmol/g in muscle.

drug-free samples at the retention time of around 18 min. The fluorescence spectra of the eluate from the forphenicine peak showed excitation and emission maxima at wavelengths of 350 and 420 nm, respectively.

The fluorescent derivative of forphenicine fluoresced most intensely in a neutral medium (pH 6.0–7.5). Therefore, phosphate buffer of pH 6.5 was added to the mobile phase. At this pH, citrate buffer or acetate buffer can also be used as an alternative. The fluorescence intensity of the eluate was approximately fifteen times that in the acidic solution of perchloric acid recommended for the derivatization.

DEB also confers fluorescence on α-keto acids (e.g., pyruvic acid, α-ketoglutaric acid, α-phenylpyruvic acid, *p*-hydroxyphenylpyruvic acid and N-acetylneuramic acid) under different conditions [9,11]. Using the present HPLC method, however, the peak heights from the above compounds were only 1–2% of that from forphenicine in equimolar amounts. Additionally, these compounds could be separated from forphenicine by the present HPLC method. Aliphatic aldehydes (formaldehyde and acetaldehyde), forphenicinol, catecholamines (epinephrine and norepinephrine), nineteen α-amino acids, reducing sugars (glucose and fructose) and steroids (estrone and estriol) did not give fluorescent derivatives under the present derivatization conditions.

The calibration graphs of peak height versus amount of forphenicine added to the serum and muscle samples were linear in the concentration ranges 0–250 nmol/ml and 0–300 nmol/g for the drug-free serum and muscle, respectively. The correlation coefficients (r) for the straight lines for serum and muscle were both 0.9998. The minimum detectable concentrations of forphenicine were 7.35 pmol/ml in serum and 5.36 pmol/g in muscle. These values corresponded to approximately 98 fmol in an injection volume of 100 μl, giving a peak height of three times the noise level.

The recoveries of forphenicine added to drug-free serum and muscle at concentrations of 2.5 and 25 nmol/ml were 97.3 ± 4.9 and 99.6 ± 4.2% in serum and 93.4 ± 5.2 and 96.5 ± 6.2% in muscle (mean $\pm$ S.D., $n = 5$), respectively.

The precision of the method for the determination of forphenicine in serum and muscle were established with six replicate assays. The relative standard deviations were 5.88 and 7.63% for the mean concentrations of 5 nmol/ml in serum and 1.5 nmol/g in muscle, respectively.

Distribution of forphenicine and forphenicinol

The concentration–time graphs for forphenicine in serum and muscle of mice dosed orally with forphenicine (30 mg/kg) are shown in Fig. 5A. The concentrations of forphenicine in both serum and muscle reached maximum values 1 h after administration and subsequently decreased at an apparent first-order rate until 8 h (the half-life was 1.9 h in serum and 3.2 h in muscle).

In the same samples of serum and muscle, forphenicinol as a metabolite of forphenicine was detected. Its concentration–time curves are also shown in Fig. 5A. In serum, the forphenicinol concentration was highest 1 h after the administration of forphenicine and then decreased slowly. In muscle, the forphenicinol concentration was lower than that in serum for the first 5 h after administration,

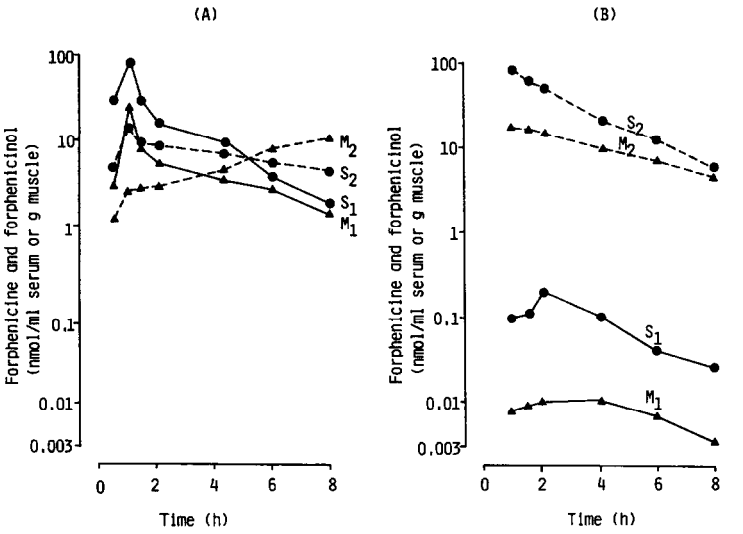

Fig. 5. Concentrations of forphenicine and forphenicinol in serum and muscle of mice ($n=3$) after oral administration of (A) forphenicine and (B) forphenicinol. Dose: 30 mg/kg each. Curves: S_1, forphenicine in serum; M_1, forphenicine in muscle; S_2, forphenicinol in serum; M_2, forphenicinol in muscle.

but its concentration increased until at least 8 h after administration. At 8 h, the concentration of the metabolite forphenicinol in the muscle was twice that in serum and approximately five times higher than the forphenicine concentration in the same serum and muscle samples.

After forphenicinol administration (Fig. 5B), the serum and muscle concentrations of forphenicinol were almost the same as those of forphenicine after administration of forphenicine. The half-life of forphenicinol was 1.8 h in serum and 3.4 h in muscle. In this instance, forphenicine was formed as a metabolite. However, the forphenicine concentrations in both serum and muscle of mice dosed with forphenicinol were much lower (100 times or less) than those of the metabolite forphenicinol in the serum and muscle of mice dosed with forphenicine.

The above results demonstrate that both forphenicine and forphenicinol can be readily distributed into the blood and muscle cells after oral administration of forphenicine.

The proposed HPLC method permits the sensitive determination of forphenicine in 100 μl of serum and ca. 0.1 g of muscle from mice dosed with either forphenicine or forphenicinol. The study has also demonstrated that forphenicine can be metabolized in the mouse body to forphenicinol and vice versa, at different metabolic rates.

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