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Liquid chromatography method for determination of mefenamic acid in human serum

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Abstract

A simple, rapid and specific method for analysis of mefenamic acid (I) in serum by a sensitive high-performance liquid chromatography is described. Only 70 µl of serum and a little sample work-up is required. A simple procedure of extraction by dichloromethane followed by evaporation to dryness under gentle stream of nitrogen and dissolving the dried residue in mobile phase was used. The mefenamic acid peak was separated from endogenous peaks on a C_8 column by a mobile phase of acetonitrile–water (50:50, v/v, pH 3). Mefenamic acid and internal standard (IS) (diclofenac) were eluted at 7.4 and 5.4 min, respectively. The limit of quantitation of mefenamic acid in serum was 25 ng/ml at 280 nm. The method was linear over the range of 25–2000 ng/ml with r^2 of 0.998. Mean recovery for mefenamic acid was 110%.
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1. Introduction

Mefenamic acid (I) ((2-[2,3-dimethylphenyl)amino]benzoic acid) (Fig. 1), an anthranilic acid derivative, is a non-steroidal anti-inflammatory drug, although it's anti-inflammatory properties are considered to be minor. It is used in mild to moderate pain including headache, dental pain, post-operative and post-partum pain and dysmenorrhoeae, in musculoskeletal and joint disorders such as osteoarthritis [1]. The need for the development of simple and still sensitive bioanalytical methods for determination of pharmacokinetic characters of drug and bioequivalence study of generic formulations is increasing. High performance liquid chromatography (HPLC) is still the most widely used method for this purpose. Some reported analytical methods for determination of mefenamic acid in serum involve time consuming and laborious extraction steps [2], lengthy retention time or use a large volume of biological sample [3,4]. Some have used fluorescence or PDA detector [4], ion pair system [5], solid phase extraction [6] or sophisticated instruments [2]. We developed a simple, rapid and applicable HPLC method which needs small sample volume and minimal sample work-up.

2. Experimental

2.1. Chemicals and reagents

All reagents were obtained from Merck (Darmstadt, Germany). Acetonitrile, dichloromethane and methanol were of HPLC grade. Phosphoric acid was of analytical grade. Double distilled water was used throughout the study. Mefenamic acid (I) and diclofenac sodium (II) as an internal standard (IS) (Fig. 1) were kindly provided by Alhavi pharmaceutical (Tehran, Iran).

2.2. Apparatus and chromatographic conditions

The chromatographic separation was performed on a model 600 solvent delivery pump, a 600E system controller, a 486 tunable absorbance detector (Waters, Millipore, Bedford, MA, USA) and a SE120 BBC recorder. Samples were introduced by a rheodyne model 7725i injector fitted by a 50 µl loop. The compounds were eluted with a mobile

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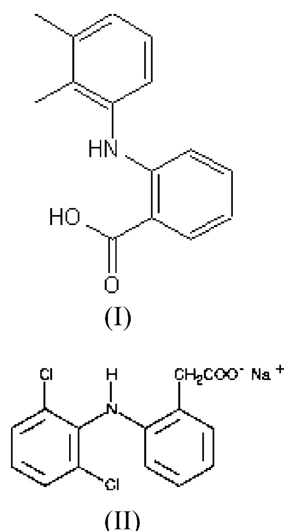

Fig. 1. Structural formula of mefenamic acid (I) and internal standard (II).

phase of acetonitrile–water (50:50), adjusted to pH of 3 by phosphoric acid. The chromatography was performed isocratically on a C_8 Techsphere column (150 mm × 4.6 mm i.d., 3 μm, HPLC Co. London, UK) at a flow rate of 1 ml/min under room temperature (25 °C). The eluents were monitored at 280 nm.

2.3. Preparation of serum calibration standards

Starting from stock solution of mefenamic acid 40 μg/ml in acetonitrile, standards were prepared using a pooled human drug free serum obtained from healthy volunteers as diluent. The calibration curve was performed with standards of the final concentrations of 25, 50, 100, 200, 250, 500, 750, 1000, 1500, 2000 and 4000 ng/ml in human serum. Working solution of diclofenac sodium (50 μg/ml) in water used as internal standard was prepared daily by dilution of stock solution of 200 μg/ml in acetonitrile.

2.4. Extraction procedure

In a 1.5 ml Eppendorf polypropylene tube, 70 μl of serum samples, 100 μl of IS and 100 μl of 1 M phosphoric acid were added and mixed for 30 s. To the solution was added 1 ml of dichloromethane, vortex mixed for 3 min and centrifuged at 10,000 rpm (8500 × *g*) for 3 min. The organic phase (700 μl) was then transferred to a clean glass tube and evaporated to dryness under N_2 stream at 45 °C. The residue was dissolved in 200 μl of mobile phase and an aliquot of 50 μl of reconstituent was injected onto HPLC column.

2.5. Human study

After overnight fasting, 12 healthy volunteers, who had given their written consent, were administered a single dose of 250 mg mefenamic acid capsule as test formulation (formulated by Alhavi Pharmaceutical) or a 250 mg Ponstan capsule as reference product (Elanpharma, UK) in a double blind cross-over bioequivalency study. The study proposal was approved by the ethical committee of Ministry of Health. Blood samples (2 ml) were taken up to 10 h after drug administration. After centrifugation, the serum part was separated and stored at −20 °C until analysis.

3. Results

Fig. 2 shows representative chromatograms of extracted serum samples for measurement of mefenamic acid. Fig. 3 shows mean concentration time profiles after administration of a single oral dose of 250 mg mefenamic acid test and reference products. The validity parameters obtained for this method are as follows.

3.1. Linearity

The linearity of the method was studied in triplicate. The calibration curve (ranged from 25 to 4000 ng/ml) was constructed by peak height ratio (mefenamic acid/IS) versus mefenamic acid concentration (ng/ml). The equation describing the linearity showed an intercept of 0.019 and a slope of 0.0003 having a correlation coefficient of 0.998. The method was specific as there was no interference between endogenous peaks and those of analyte and IS in chromatograms.

3.2. Recovery

Extraction recovery of mefenamic acid from serum was assessed by calculating the ratio of the peak height of analyte in serum extract and aqueous solution. An average recovery of 110 ± 7.5% (mean ± S.D.) was obtained in concentrations (50, 1000 and 2000 ng/ml) studied ($n = 5$).

3.3. Inter and intra-day assay precision and accuracy

The results of the assay in one and five different days showed a mean R.S.D. of 10.6 and 11.7% for intra and inter-day reproducibility, respectively (Table 1).

3.4. Sensitivity

The limit of quantitation (LOQ) of the method as signal/noise of 5 was equal to 25 ng/ml. Considering a signal/noise of 3, a LOD of 15 ng/ml was determined.

4. Discussion

The results obtained from our method development show that this method has some advantages over published methods. The column switching HPLC method described by Yamashita et al. [2], in spite of its very low and interesting

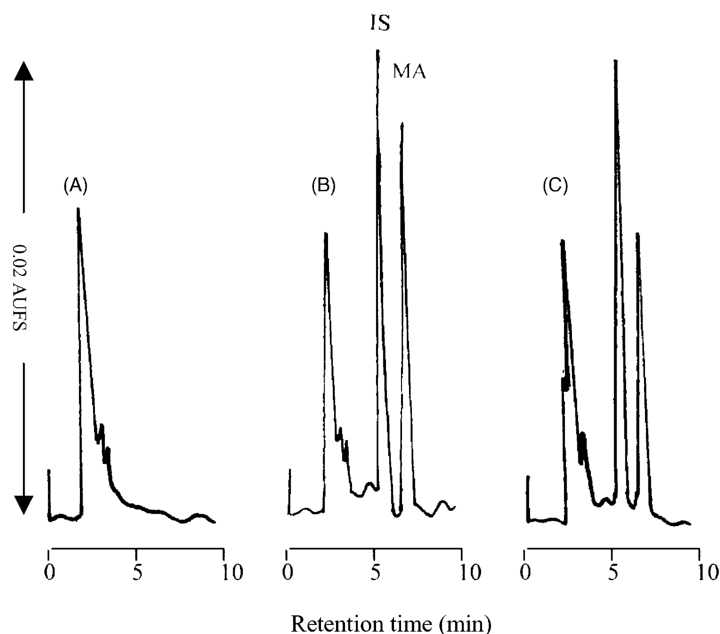

Fig. 2. Chromatograms of (A) blank serum; (B) spiked serum by IS and mefenamic acid (retention times IS: 5.4 min, mefenamic acid: 7.4 min); and (C) serum from a volunteer 3 h after oral administration of 250 mg mefenamic acid capsule.

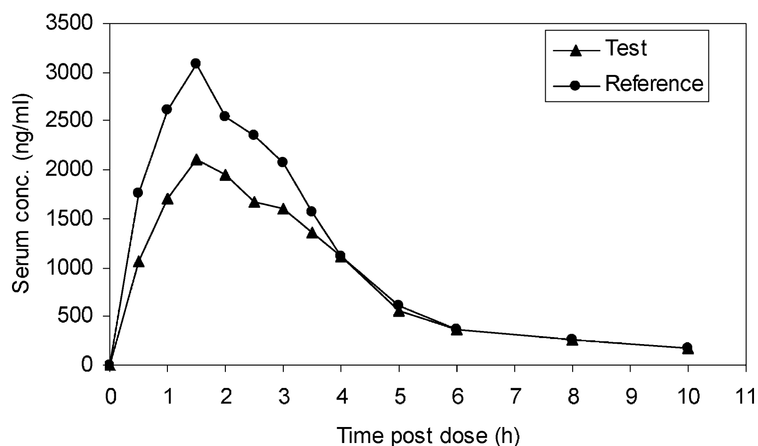

Fig. 3. Mean serum concentration time profile of mefenamic acid after a single oral administration of 250 mg of mefenamic acid test or reference products to 12 healthy volunteers.

LOD, is expensive and not applicable in all labs. Singh et al. [4] have developed an HPLC method using fluorescence detection. Although this method has almost the same sensitivity as ours (LOD 20–50 ng/ml), but it needs one ml biologic sample. In another study, Streete [7] used a direct protein precipitation method for sample preparation. This method is rapid and simple, however, it is able to determine only high therapeutic concentrations. Niopas and Mamzoridi [8] also have published a method which has a LOD of about 60 ng/ml. The method published by Maron

Table 1
Intra and inter-day assay accuracy and precision for the determination of mefenamic acid in human serum ($n = 5$)

Amount added (ng/ml)	Intra-day assay			Inter-day assay		
	Amount found (ng/ml)	R.S.D. (%)	Bias (%)	Amount found (ng/ml)	R.S.D. (%)	Bias (%)
100	91.3	9.5	8.7	90.8	12.2	9.2
200	194.2	9.2	6.9	209.0	12.0	−4.5
400	429.2	10.6	−7.3	432.8	11.2	−8.2
600	631.2	10.4	5.2	553.8	11.7	7.7

and Wright [9] has also a simple sample work-up, but this method suffers from lengthy retention time (≅20 min).

5. Conclusion

The described method here has a short run time, needs small volume of serum and has an acceptable sensitivity. It seems that this method is suitable for pharmacokinetic and bioequivalency studies in all laboratories equipped with unsophisticated instruments.

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