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Validation of the HPLC method for model determination of fenoprofen in conjugates with PHEA

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An isocratic RP HPLC method for model determination of fenoprofen in PHEA-fenoprofen conjugates was developed and fully validated. The proposed method is a modification of the official HPLC method for fenoprofen in respect of the type of the stationary phase and mobile phase composition. The method was found to be specific, precise (*RSD* 0.9%), accurate (mean recovery 99.2%) and robust. Limit of detection was estimated at 0.02 μg mL^{-1} and the limit of quantification at 0.09 μg mL^{-1}.

Keywords: fenoprofen, PHEA-fenoprofen conjugate, determination, HPLC, validation

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Fenoprofen, a potent nonsteroidal anti-inflammatory drug, has been widely used in the treatment of rheumatoid arthritis, gout, osteoarthritis, and acute muscle-skeletal disorders (1). Unfortunately, like other nonsteroidal anti-inflammatory agents, fenoprofen carries the risk of side-effects, such as gastrointestinal irritation and ulceration. To overcome these problems, numerous fenoprofen prodrugs of ester and amide type have been synthesised and tested for their analgesic/anti-inflammatory activity and gastrointestinal compliance (2–5). Our research is directed towards polymer-fenoprofen conjugates, in which fenoprofen is covalently bound to hydrophilic polymers of polyaspartamide type. In our previous papers, syntheses of poly[α,β-(*N*-2-hydroxyethyl-DL-aspartamide)] (PHEA) fenoprofen conjugates and several related conjugates were described (6, 7). In order to quantitate fenoprofen released from PHEA-fenoprofen conjugates (8), a reliable analytical method for fenoprofen determination was crucial. For these purposes, a modified USP 24 (9) HPLC method for fenoprofen was used. Since the original scope of this official method was changed in regard to both method parameters and applicability, relevant validation had to be performed and will be discussed in this paper. The outcome of such validation contributes to the knowledge of the application possibilities of the modified method.

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EXPERIMENTAL

Chemicals

Fenoprofen was purchased from Eli Lilly (South Africa). PHEA was prepared by thermal polycondensation of L-aspartic acid and subsequent aminolysis with ethanolamine (10). The syntheses of PHEA-fenoprofen conjugates, differing in type of covalent bonding, type and/or length of spacer, drug-loading and polymer average molecular mass, were described previously (6–8).

All solvents were of HPLC grade quality (Merck, South Africa).

Apparatus and methods

Fenoprofen standard solutions were prepared by dissolving fenoprofen calcium in water to cover a concentration range 66.6–150.0 $\mu g\ mL^{-1}$ of fenoprofen calcium, equivalent to 28.9–65.1 $\mu g\ mL^{-1}$ of fenoprofen.

The HPLC system (Thermo Separations, USA) consisted of a spectraSYSTEM AS 3000 autosampler with a variable volume loop injector, a spectraSYSTEM P1000 isocratic pump, a spectraSYSTEM SN 4000 signal converter and a spectraSYSTEM UV 1000 programmable variable wavelength detector set at 272 nm, with a 10-mm analytical flow cell. Phenomenex Luna C_{18} and C_8 columns (Separations, South Africa) were used comparatively. The mobile phase was acetonitrile/water 3:2, adjusted to pH 2 with concentrated phosphoric acid. The mobile phase was flushed through the column at a flow rate of 1.75 mL min^{-1} at room temperature (25 ± 2 °C). The injection volume was 20 μL.

Peak areas were used to quantify fenoprofen in the various samples. Limit of detection (LOD) and limit of quantification (LOQ) values were estimated using an average *SD* obtained by repetitive measurements ($n = 6$) of fenoprofen in the concentration range 0.1–0.3 $\mu g\ mL^{-1}$. This mean *SD* was divided by the slope of the standard curve and multiplied by factor 3 to obtain the LOD and factor 10 to obtain the LOQ, respectively.

Raw data were processed by means of a Pentium MMX 166 MHz computer and TSP PC 1000 integration software package operating in an IBM OS/2 Warp version 3 environment.

RESULTS AND DISCUSSION

The paper deals with the experimental details necessary for the selection of a suitable chromatographic system for the quantification of fenoprofen in a particular system, *e.g.*, in PHEA-fenoprofen conjugates and in kinetic studies of fenoprofen release from the conjugates. Validation was done according to the protocol suggested by the United States Food and Drug Administration guidelines for chromatographic method validation (11).

Chromatographic system

Chromatographic parameters such as the capacity factor, peak symmetry and theoretic plate count were utilised to evaluate the chromatography and column performance. The selection of a suitable column was guided by the HPLC method described in the monograph for fenoprofen calcium in the USP 24 (9). Although the official method utilises a C_{18} column, a C_8 column was also included for comparative and optimising purposes. The Luna brand columns were selected so as to employ the latest advances in silica and bonding technology. The silica used is ultra pure and has remarkable surface smoothness, improving the stability and column efficiency (12). The column is also known for its pH stability, improved resolution and resistance to acids and bases. The particulars of the columns used are given in Table I.

Table I. Particulars of the Luna columns used

Particular	Column	
	Luna 5 μ C_{18}	Luna 5 μ C_8
Dimensions (mm)	150 × 4.6	
Particle size (μm)	4.90	
Particle distribution (90/10%)	1.96	
Pore diameter (10^{-10} m)	100	
Surface area ($m^2\ g^{-1}$)	405	
Total carbon content (%)	17.89	14.26
Surface coverage (μmol m^{-2})	3.44	4.65

In order to select the most suitable column (C_{18} or C_8), a standard solution of fenoprofen calcium was chromatographed on both columns. The resulting chromatograms were evaluated and compared using the performance parameters given in Table II.

Due to its slightly better peak symmetry, shorter retention time, higher N and smaller HETP, the C_8 column was selected for further validation and optimisation of the HPLC method for fenoprofen. To optimise the separation efficiency and peak symmetry, the composition and flow rate of the mobile phase were slightly altered from the USP

Table II. Comparison of performance data between C_{18} and C_8 columns during fenoprofen chromatographing

Performance parameter	Column	
	Luna 5 μ C_{18}	Luna 5 μ C_8
Capacity factor	10.20	8.70
Retention time (min)	3.20	2.80
Peak symmetry factor	1.42	1.20
Plates/meter	50800	52580
HETP (mm)	0.02	0.01

official method (9). The mobile phase used consisted of acetonitrile/water 3:2, adjusted to pH 2 with concentrated phosphoric acid and the flow rate was 1.75 mL min^{-1}, while the USP procedure used acetonitrile/water/phosphoric acid in the volume ratio 50:49.6:0.4 with a flow rate of 2 mL min^{-1}.

Performance characteristics

Specificity. – Fenoprofen was spiked with 1 mg mL^{-1} solution of PHEA (the polymeric carrier in PHEA-fenoprofen conjugate) at five concentration levels ranging from 29 to 65 μg mL^{-1}. PHEA/fenoprofen mass ratio ranged from 15:1 to 35:1. Agreement of the results of 100.9 ± 0.9% (range 98.8–103.0%) indicates the absence of PHEA interference, *e.g.*, the method was found specific for fenoprofen in the presence of PHEA. A chromatogram representing the spiked fenoprofen solution is shown in Fig. 1. Only the fenoprofen peak is visible since PHEA does not migrate in the selected chromatographic system and shows no UV absorption between 250–300 nm.

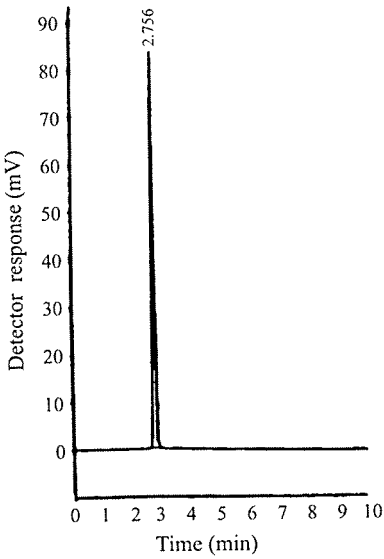

Fig. 1. Chromatogram of the PHEA-spiked fenoprofen solution (fenoprofen concentration levels ranged from 29 to 65 μg mL^{-1}; PHEA/fenoprofen mass ratio ranged from 15:1 to 35:1).

No interference from any degradation product of fenoprofen (13) and PHEA (8) was expected, since both substances were found to be stable under the experimental conditions. According to our results (8), no degradation products were detectable by HPLC after treating fenoprofen or PHEA with the mobile phase for several days.

Linearity and sensitivity. – Linearity was determined using a series of five fenoprofen concentrations ranging between 80–120% of the expected concentration, *e.g.*, in the concentration range from 29 to 65 μg mL^{-1}. Peak area (mV s) *versus* concentration (μg mL^{-1})

data fit to the regression line: $y = 6767.4\gamma - 34917$ (multiple $R = 0.999$, $R^2 = 0.999$, adjusted $R^2 = 0.998$, 19 observations).

Precision. – Injection repeatability of 0.2 to 0.4% ($n = 5$) and intra-assay precision of 0.9% ($n = 6$) were estimated using a fenoprofen sample of $\gamma = 50\ \mu g\ mL^{-1}$. Both precision data were within the recommended precision criteria (10, 14).

Accuracy. – The accuracy data set is presented in Table III. The average recovery of fenoprofen ranged from 99.5 to 100.4% when the mobile phase of pH 2.0 was used. The method can thus be regarded as accurate.

Table III. Accuracy and robustness data set

Target conc. (%)	*Spiked conc.* ($\mu g\ mL^{-1}$)	*Mean recovery (%)*[a]		
		pH 1.8	pH 2.0	pH 2.2
66	28.64	97.1	100.2	99.8
80	34.72	100.4	99.5	100.6
100	43.40	101.0	100.0	99.8
120	52.08	100.4	100.4	101.3
150	65.10	99.1	100.1	99.8

[a] Number of replicates, $n = 6$.

Ruggedness and robustness. – To determine the ruggedness of the method, the analysis was performed on two different instruments (H1 and H2) and on different days. Each instrument performed well with respective precision of 0.8 and 0.9%, recovery of 99.2 and 98.9% and linearity correlation coefficient of 0.998 for both instruments. Intermediate precision of 1.0% was calculated as a measure of overall precision on different days.

Robustness was tested by deliberately changing the pH of the mobile phase from 2.0 to 1.8 and 2.2 (Table III) (pH lower than 1.8 and higher than 2.2 caused peak distortion and produced unacceptable chromatograms). Recoveries obtained at pH 1.8, 2.0 and 2.2 were estimated at 99.6 ± 1.6, 100.1 ± 0.3 and 100.2 ± 0.7, respectively. It is evident that favourable recovery values were achieved at all the tested pH values. The method has proven robust in respect to slight pH changes.

Limiting values. – The limit of detection for fenoprofen using the described HPLC system was 0.02 $\mu g\ mL^{-1}$. The concentration that could be quantitatively determined with acceptable accuracy and precision was 0.09 $\mu g\ mL^{-1}$. These LOD and LOQ values will be very important if future *in vivo* studies of the PHEA-fenoprofen conjugates are to be undertaken utilising this HPLC method.

CONCLUSIONS

The proposed method for fenoprofen determination is a modification of the known method in respect of the stationary phase type and mobile phase composition. The most suitable chromatographic system (including: C_8 stationary phase, acetonitrile/water 3:2

adjusted to pH 2.0, flow rate 1.75 mL min^{-1}) was subjected to extensive validation in order to assure that all data obtained using the newly modified analytical method were reliable. Even under deliberate variations in method parameters, like spiked contaminants, mobile phase pH changes, different instruments and time intervals, the method still proved to be highly precise, accurate and specific. The proposed method has already proven useful in kinetic studies of fenoprofen release from PHEA-conjugates *in vitro* (8). One could expect that the method might form a basis for fenoprofen determination in biological fluids as well.

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SAŽETAK

Validacija HPLC metode za modelno određivanje fenoprofena u konjugatima s PHEA

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Razvijena je i validirana izokratična RP HPLC metoda za modelno određivanje fenoprofena u PHEA-fenoprofen konjugatima. Predložena je modifikacija službene HPLC metode za fenoprofen primjenom druge stacionarne faze i promijenjenog sastava mobile faze. Metoda se pokazala specifičnom, preciznom (*RSD* 0,9%), ispravnom (srednji analitički povrat 99,2%) i robustnom. Granica detekcije iznosi 0,02 $\mu g\ mL^{-1}$, a granica kvantifikacije 0,09 $\mu g\ mL^{-1}$.

Ključne riječi: fenoprofen, PHEA-fenoprofen konjugat, određivanje, HPLC, validacija

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