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Determination and validation of ketoprofen, pantoprazole and valsartan together in human plasma by high performance liquid chromatography

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Received June 15, 2005, accepted September 12, 2005

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Pharmazie 61: 586–589 (2006)

A rapid and specific high-performance liquid chromatographic method was developed and validated for the simultaneous determination of ketoprofen, valsartan and pantoprazole in human plasma. Chromatographic separation of ketoprofen, valsartan and pantoprazole was performed using a Chromasil C_{18} column (250 mm × 4.6 mm i.d., 5 μm particle size). The mobile phase consisted of a mixture of 0.02 M sodium dihydrogen phosphate buffer (pH 3.15) and acetonitrile (58 : 42, v/v) pumped through the chromatographic system at a flow rate of 1 $mL \cdot min^{-1}$. The Diode Array detector was operated at 225 and 272 nm. Rofecoxib was used as an internal standard. Sample treatment procedure consisted of deproteinisation with acetonitrile-methanol (50 : 50 v/v). Analytical recoveries were in the range of 79.00–118.00% of nominal values of valsartan, ketoprofen and pantoprazole. The method was reproducible and accurate with lower limits of quantification 250 $\mu g \cdot L^{-1}$ for pantoprazole and 500 $\mu g \cdot L^{-1}$ for ketoprofen and valsartan. This method was relatively easy to perform and allows simultaneous determination of these three drugs in plasma at nanogram levels.

1. Introduction

Many patients are likely to receive both antihypertensives and nonsteroidal anti-inflammatory drugs (NSAIDs), or NSAIDs and proton pump inhibitors, or even these three drugs together. Monitoring combinations of two or three of these drugs in plasma helps to improve the effectiveness of therapy by minimizing drug toxicity and ensuring an appropriate dosage regimen (Kshirsagar 1994).

Most of the analytical methods found in the literature use HPLC to determine ketoprofen (Dvořák et al. 2004; Martin et al. 1999), valsartan (Şatana et al. 2001; González et al. 2002; Daneshtalab et al. 2002) and pantoprazole (Cass et al. 2001) are aimed at quantifying these drugs in biological fluids for post mortem analysis to determine the raw material and its related substances. However, no methods have been described using LC for the three drugs together in biological fluids. The aim of this study was to develop and validate a LC method to quantity ketoprofen, valsartan and pantoprazole together in biological fluids.

2. Investigations and results

Typical chromatograms of blank human plasma, and plasma spiked with LLOQ values of ketoprofen, pantoprazole and valsartan in the presence of the internal standard are shown in the Fig. To avoid interferences and maximize the sensitivity of the assay, we chose detection wavelengths of 225 nm for valsartan and 272 nm for ketoprofen and pantoprazole. There was no interference in drug-free plasma at retention times of interest. Under the chromatographic conditions described, retention times were approximately 5.8, 12.1, 16.7 and 20.6 min for pantoprazole, rofecoxib, ketoprofen and valsartan, respectively.

The calibration data and calibration curve parameters (Table 1) demonstrate that the calibration curves were linear in the concentration range from 500 μg to 8 $mg \cdot L^{-1}$ for ketoprofen and valsartan, and 250 μg to 4 $mg \cdot L^{-1}$ for pantoprazole. The linear regression equation was $y = 0.0008x - 0.0802$ with a correlation coefficient of 0.9944 for ketoprofen, $y = 0.0005x - 0.0503$ with a correlation coefficient of 0.9962 for pantoprazole and $y = 0.0005x - 0.0191$ with a correlation coefficient of 0.9943 for valsartan.

Potential interferences with the determinations were investigated by assaying blank plasma spiked with ketoprofen, pantoprazole and valsartan. No significant chromatographic interferences were found with the three drugs tested and internal standard.

The results for accuracy and within- and between-run precision for samples are presented in Table 2. The within-run precision showed R.S.D.s of 1.47–7.11%, 6.44–10.06% and 4.76–9.66% for valsartan, pantoprazole and ketoprofen respectively. The between-run precision similarly evaluated over four consecutive days at three different concentrations varied from 2.73–7.15% for valsartan, 13.46–14.26% for pantoprazole and 8.87–11.99% for ketoprofen.

The lower limits of quantification (LLOQs) were 500 $\mu g \cdot L^{-1}$ for ketoprofen and valsartan, and 250 $\mu g \cdot L^{-1}$ for pantoprazole.

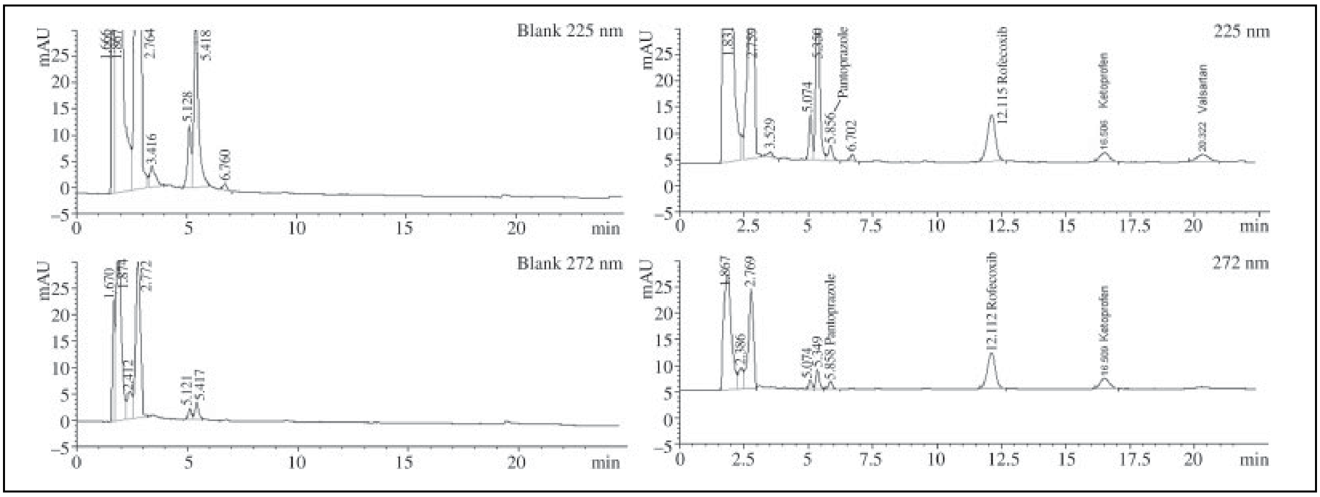

Fig.: Typical chromatograms of blank sample and spiked sample with pantoprazole (250 μg · L^{-1}), ketoprofen (500 μg · L^{-1}), valsartan (500 μg · L^{-1}) and 1.25 mg · L^{-1} rofecoxib (I.S.). Mobile phase: 58% 0.02 M sodium dihydrogen phosphate buffer, pH 3.15 and 42% acetonitrile (v/v) at a flow-rate of 1 mL · min^{-1}. Diode Array detector: 225 nm and 272 nm

Table 1: Reproducibility of daily (n = 3) calibration curves

Analysis day	Slope	Intercept	Correlation coefficient
Valsartan			
1	0.0005	−0.0258	0.9901
2	0.0006	−0.1383	0.9985
3	0.0006	−0.0833	0.9943
Mean	5.7E-04	−0.0191	0.9943
S.D (±)	5.8E-05	0.0563	0.0042
R.S.D.(%)	10.19	−68.22	0.42
Pantoprazole			
1	0.0006	−0.1092	0.9982
2	0.0005	−0.0325	0.9978
3	0.0006	−0.0092	0.9927
Mean	5.7E-04	−0.0503	0.9962
S.D (±)	5.7E-05	0.0523	0.0031
R.S.D. (%)	10.19	−104.02	0.41
Ketoprofen			
1	0.0008	−0.0875	0.9952
2	0.0008	−0.1088	0.9957
3	0.0009	−0.0442	0.9924
Mean	8.3E-04	−0.0802	0.9944
S.D (±)	5.7E-05	0.0329	0.0018
R.S.D.(%)	6.93	−41.06	0.24

Table 2: Precision and accuracy

	Conc. added (μg · L^{-1})	Mean conc. found (μg · L^{-1})	Recovery (%)	Accuracy (%)	Precision (%)	n
Reproducibility on same day						
Valsartan	0.50	0.56	112.00	12.00	1.47	4
Pantoprazole	0.25	0.20	80.00	20.00	9.59	4
Ketoprofen	0.50	0.46	92.00	8.00	6.54	4
Valsartan	2.00	1.63	81.50	18.50	6.35	4
Pantoprazole	1.00	0.82	82.00	18.00	10.06	4
Ketoprofen	2.00	1.58	79.00	21.00	9.66	4
Valsartan	8.00	7.22	90.25	9.75	7.11	4
Pantoprazole	4.00	3.49	87.25	12.75	6.44	4
Ketoprofen	8.00	7.10	88.75	11.25	4.76	4
Day-to-day reproducibility						
Valsartan	0.50	0.55	110.00	10.00	7.15	4
Pantoprazole	0.25	0.26	104.00	4.00	14.26	4
Ketoprofen	0.50	0.59	118.00	18.00	8.87	4
Valsartan	2.00	1.85	92.50	7.50	3.47	4
Pantoprazole	1.00	0.93	93.00	7.00	13.73	4
Ketoprofen	2.00	1.96	98.00	2.00	11.99	4
Valsartan	8.00	8.39	104.87	4.87	2.73	4
Pantoprazole	4.00	3.95	98.75	1.25	13.46	4
Ketoprofen	8.00	8.06	99.25	0.75	9.23	4

The recovery was studied by adding valsartan, pantoprazole and ketoprofen at three different concentrations to blank plasma samples and analyzing four replicates over four days. Recoveries determined ranged from 79.00–118.00% of the nominal values of valsartan, pantoprazole and ketoprofen.

3. Discussion

In this study, a reverse-phase LC method with DAD detection was developed and validated for the simultaneous quantitation of pantoprazole, ketoprofen and valsartan in human plasma. Initially various mobile phase compositions were tried to separate these three drugs and rofecoxib, the internal standard, on a C_{18} column in an isocratic system. A mixture of 58% phosphate buffer solution (pH 3.15) and 42% acetonitrile was then selected as the mobile phase to give good separation. The peaks corresponding to the compounds used in this study were well separated and were sharp and symmetrical. The advantage of the method used in this study was the precipitation of proteins without an extraction step such as liquid-liquid extraction (Daneshtalab et al. 2002; Woolf et al. 1999; Reddy et al. 2002). Protein precipitation with acetonitrile-methanol (50 : 50 v/v) allowed the simultaneous determination of these three drugs together in plasma.

The results of validation of the method demonstrated excellent precision and accuracy with acceptable specificity and chromatographic resolution. Good linearity in terms of peak area ratios as a function of analyte concentration coefficients was observed for the regression lines. Rofecoxib was regarded as an acceptable internal standard because it exhibits similiar extraction and chromatographic properties to the analyte.

Recoveries were in the range of 79–118% of nominal values of valsartan, pantoprazole and ketoprofen. The relative standard deviations for three different concentrations of valsartan, pantoprazole and ketoprofen in plasma in a same-day study varied from 3.48–5.10%, 4.03–5.98% and 3.17–6.99% respectively, whereas those in the day-to-day study varied from 2.73–7.15%, 13.46–13.73% and 8.87–11.99% respectively. The precision and accuracy should always be within ±15% for bioanalytical methods except at the lower limit of quantification, where they should not deviate by more than 20% (Shah et al. 1992).

In conclusion, the chromatographic conditions established and validated in this study were simple, easily reproducible and sufficiently specific to separate ketoprofen, pantoprazole, valsartan and rofecoxib (I.S) from other components in plasma samples. The present method is relatively easy to perform and allows the simultaneous determination of these three drugs together in plasma at low levels. This method can also be used in a hospital laboratory for therapeutic drug monitoring of these drugs in patients plasma.

4. Experimental

4.1. Equipment

The chromatographic system used to develop this technique was a Hewlett Packard 1100 featuring a column oven (G1316 A), a quaternary pump (G 1311 A), a manual injector (G 1328 B) and DAD detector (G 13115 B) which is set at 225 and 272 nm. Data acquisition was performed using a chromatography software package (Agilent Chemstation version 9.01(1206)).

4.2. Materials

The acetonitrile used was HPLC grade, supplied by Merck. Sodium dihydrogen phosphate was supplied by Fluka. Ortho-phosphoric acid 85%, for analysis was purchased from JT Backer. All the water used was purified by the Milli-Q-Grade water system (Millipore).

Ketoprofen (98% purity) and pantoprazole (99% purity) were kindly supplied by Nobel Pharm. Ind and İlsan İltaş Pharm. Ind. (Istanbul, Turkey). The internal standard, rofecoxib (99% purity), was procured from Sanovel Pharm. Ind (İstanbul, Turkey). Valsartan was isolated from the tablet form using an extraction method (Şatana et al. 2001): Ten tablets containing valsartan as active ingredient were finely powdered, diluted with methanol and sonicated for 5 min. After centrifugation, the liquid phase was transferred to a clean tube and then evaporated under vacuum. The residue was washed with hot water and dried.

4.3. Chromatographic conditions

Chromatographic separation of valsartan, pantoprazole, ketoprofen and internal standard were performed using a Chromasil C_{18} 250 mm × 4.6 mm i.d. column, made of stainless steel. The mobile phase consisted of 58% 0.02 M sodium dihydrogen phosphate buffer, pH 3.15 and 42% of acetonitrile. To prepare the buffer solution, 3.12 g of dihydrogen sodium phosphate was dissolved in about 1000 mL of HPLC grade water. Once dissolved, the pH was adjusted to 3.15 ± 0.05 with ortho-phosphoric acid, reactive analysis. Both the acetonitrile and the buffer solution were filtered though a 0.45 μm GH-membrane filter. The mobile phase was pumped through the chromatographic system at a flow rate of $1\ \text{mL} \cdot \text{min}^{-1}$. The Diode Array detector was operated at 225 and 272 nm. The volume injected into the chromatographic system was 20 μL. Each test required 23 min.

4.4. Preparation of standard solution

Stock solutions containing ketoprofen, pantoprazole and valsartan were prepared in acetonitrile and methanol respectively at a concentration of $1\ \text{g} \cdot \text{L}^{-1}$. Stock solutions were stored at −20 °C.

Standard solutions for calibration were prepared by diluting stock solutions with blank plasma to final concentrations of $250\ \mu\text{g}–4\ \text{mg} \cdot \text{L}^{-1}$ for pantoprazole and $500\ \mu\text{g}–8\ \text{mg} \cdot \text{L}^{-1}$ for ketoprofen and valsartan. Rofecoxib as internal standard was dissolved in acetonitrile and the stock solution of rofecoxib diluted with acetonitrile-methanol (50 : 50 v/v) to a concentration of $1.25\ \text{mg} \cdot \text{L}^{-1}$.

4.5. Sample preparation

Drug-free human plasma samples were provided by the Haydarpasa Numune Hospital of İstanbul, Turkey. A 200 μL plasma sample was mixed with 200 μL of a solution of rofecoxib $1.25\ \text{mg} \cdot \text{L}^{-1}$ in acetonitrile-methanol (50 : 50 v/v) using a vortex mixer and ultrasound. Subsequently, the mixture was centrifuged at 10000 rpm and at 4 °C for 15 min. The supernatant (300 μL) was evaporated under a stream of nitrogen at 45 °C. The residue was reconstituted in 40 μL acetonitrile-methanol (50 : 50 v/v) and directly injected into the LC column under the optimised chromatographic conditions.

4.6. Validation study

4.6.1. Calibration curves

A peak area ratio method was used to calculate the concentrations of ketoprofen, valsartan and pantoprazole in reference to rofecoxib as an internal standard. Standard solutions of ketoprofen, valsartan and pantoprazole were used to spike human plasma in order to obtain calibration standards at concentrations of $500\ \mu\text{g}–8\ \text{mg} \cdot \text{L}^{-1}$ for ketoprofen and valsartan and $250\ \mu\text{g}–4\ \text{mg} \cdot \text{L}^{-1}$ for pantoprazole. The calibration standards were prepared daily. Calibration curves were obtained by plotting the peak area ratios of all three drugs to the internal standard rofecoxib at a concentration of $1.25\ \text{mg} \cdot \text{L}^{-1}$. The regression equations were obtained by the least-squares method using a regression analysis. Calibration curve parameters are presented in Table 1. The accuracy and precision of the method were determined at concentrations 500 μg, 2 mg and $8\ \text{mg} \cdot \text{L}^{-1}$ for ketoprofen and valsartan, 250 μg, 1 mg and $4\ \text{mg} \cdot \text{L}^{-1}$ for pantoprazole in plasma.

4.6.2. Linearity

From the recorded peak areas, the ratios of drug to internal standard were calculated. Unweighted least-squares linear regression of the peak area ratio as a function of the theoretical concentrations was applied to each standard curve ($y = ax + b$, where x = concentration ($\text{mg} \cdot \text{L}^{-1}$), y = peak area ratio, a = slope, and b = intercept).

4.6.3. Selectivity

The method was checked to ensure that there was no interference with valsartan, pantoprazole and ketoprofen from matrix co-extractives. Chromatograms showed that peaks attributable to these three drugs and the internal standard were resolved sufficiently from other peaks to enable reliable quantification.

4.6.4. Precision and accuracy

The precision and accuracy of this LC method were established by repetitive analysis of samples in plasma against a calibration curve. Each sample at three concentration levels was analyzed four times consecutively within one day ($n = 4$) to determine within-run precision and accuracy, and once a day for four successive days ($n = 4$) at three concentration levels to determine between-run precision and accuracy. Accuracy was expressed as the recovery (100 × measured concentration/theoretical concentration), while the precision was given by the between- and within-run relative standard deviations (R.S.D.s).

4.6.5. Determination of the limits of quantitation

The lower limit of quantitation (LLOQ) was derived from multiple measurements in the low concentration range and was determined based on the signal-to-noise approach. The level of approximately ten times the noise level was used as the LLOQ, which is the lowest concentration point in the calibration curve (FDA/CDER guidance, Bioanalytical Method Validation, May 2001).

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