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Development and validation of a new reversed-phase ion pairing liquid chromatographic method with fluorescence detection for penciclovir analysis in plasma and aqueous humor

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Abstract

A simple, sensitive and reliable HPLC ion-pairing method with fluorescence detection, was developed for penciclovir determination in plasma and aqueous humor, with a Zorbax SB-aq C18 (100 mm × 2.1 mm) column. Plasma samples were treated by solid-phase extraction with Oasis® MCX (30 mg) cartridges. Ganciclovir, an antiviral drug structurally related to penciclovir, was used as internal standard (I.S.). Aqueous humor samples were directly injected into the chromatographic system. Separation was performed by a gradient elution with a mobile phase consisting of a mixture of acetonitrile and phosphate buffer 50 mM containing 5 mM of sodium octanesulfonate, pH 2.0, at a flow rate of 0.3 ml/min.

The method was validated and showed good performances in terms of linearity, sensitivity, precision and trueness. Quantification limit was obtained at 0.05 μg/ml for aqueous humor and at 0.1 μg/ml for plasma. Finally, the proposed analytical method was used to measure penciclovir in clinical samples for a pharmacokinetic study, after oral administration of famciclovir.

Keywords: Penciclovir; Liquid chromatograpy–fluorescence detection; Plasma; Aqueous humor; Validation

1. Introduction

Penciclovir, a metabolite of the oral prodrug famciclovir, is a nucleoside analogue active against *herpes simplex virus type I and II* (*HSV*), *varicella zoster virus* (*VZV*) and *Epstein-Bar virus* (*EBV*) [1–3]. It has been demonstrated that, in ocular herpes zoster, penciclovir reduced the incidence of ocular complications [4], but ocular penetration and pharmacokinetics of penciclovir in the human eye are still unknown.

After oral administration, famciclovir is converted to penciclovir by the removal of two ester groups and the 6-oxidation of the deoxyguanine ring. Four intermediate metabolites depending on the rate of de-esterefication and oxidation are obtained [5,6], but pharmacokinetic studies have shown that penciclovir, and to a minor extend BRL42355, the desacetyl non-oxidized metabolite, are predominant in plasma and urine [7,8].

Penciclovir, like aciclovir and ganciclovir, is a nucleoside analogue that shares a structural similarity with endogenous compounds (Fig. 1). Therefore, selective analytical methods are required to analyse these compounds in biological fluids [9]. In previous pharmacokinetic studies [7,8,10,11], analysis of penciclovir was performed by high-performance liquid chromatography (HPLC) and UV detection [6,12]. However, absolute recovery was about 50–65% and the limit of quantification was of 0.2 μg/ml, which is not sensitive enough for carrying out pharmacokinetic studies. Fluorescence detection can overcome this limitation since penciclovir, like other nucleoside analogues, is natively fluorescent under acidic conditions. Moreover, since nucleoside analogues are very polar, addition of ion pairing agents or organic modifiers (i.e. triethylamine) in the mobile phase has been used to increase their retention in reversed phase

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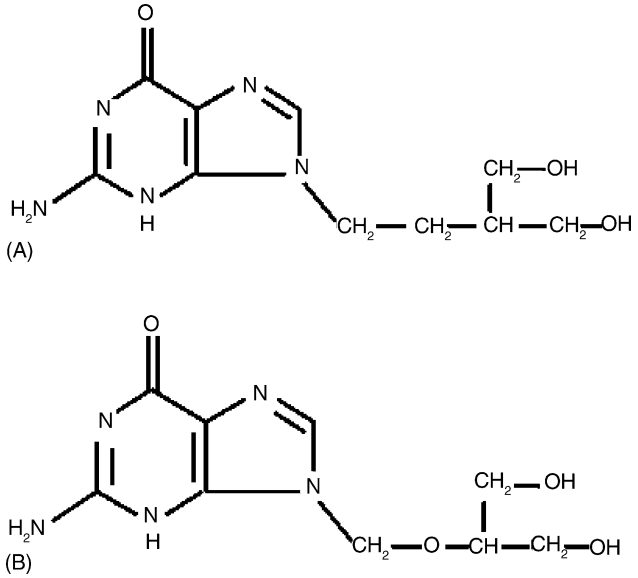

Fig. 1. Structures of penciclovir (A) and ganciclovir (I.S.) (B).

chromatography [9]. Penciclovir has also been analysed with capillary electrophoresis (CE) coupled with an UV detection [13]. However, in spite of its high selectivity, efficiency and fast separation time, this technique suffers from low sensitivity. For other purine nucleoside analogues, such as aciclovir and ganciclovir, HPLC, CE and other immunoassay methods have also been developed [9,14–40].

For the analysis of plasma, samples must be purified before chromatography to eliminate interfering compounds. Solid-phase extraction has become a well-established sample preparation technique in bioanalytical applications. Development for polar compounds has received most attention because retention problems are encountered with these compounds when using the classical silica or polymeric SPE sorbents [41]. Recently, a new polymeric mixed-mode cation-exchange and reversed phase sorbent (Oasis® MCX) was developed to enhance selectivity of polar and basic compounds such as penciclovir. Moreover, in addition to the pH stability common to polymers, Oasis® MCX has a greater binding capacity than other silica mixed-mode SPE sorbents [42]. Furthermore, the hydrophilic properties of these new cartridges obviated the difficulty of maintaining moisture, a problem that is encountered with the silica based C18 columns, and therefore reproducibility was obtained even when the cartridges had run dry [43].

The main purpose of this work was to develop a rapid, selective and sensitive method for penciclovir determination in plasma and aqueous humor. In order to enhance method selectivity, plasma samples were purified by solid-phase extraction using a polymeric mixed-mode sorbent and an ion-pairing agent was added to the mobile phase. Furthermore, fluorimetric detection was used to improve sensitivity. The method was evaluated in terms of recovery, linearity, precision and trueness, and applied to the analysis of human plasma and aqueous humor samples.

2. Experimental

2.1. Chemicals and reagents

Penciclovir was kindly provided by Smithkline Beecham Pharmaceuticals (Worthing, West Sussex). Ganciclovir was purchased from Fluka (Buchs, Switzerland). Blank human plasma and aqueous humor were obtained from the blood transfusion facilities and the Ophthalmology Service, respectively (University Hospitals, Geneva, Switzerland). HPLC grade methanol, acetonitrile, sodium octane sulfonate, sodium hydroxide, orthophosphoric acid 85%, hydrochloric acid 37%, and ammonia solution 25% were purchased from Fluka (Buchs, Switzerland). To prepare buffers and solutions, ultra-pure water was supplied by a Milli-Q purification unit from Millipore (Bedford, MA, USA).

2.2. Apparatus

The chromatographic equipment consisted of an Agilent 1100 Series LC system (Agilent, Paolo Alto, USA) with a quaternary pump, a vacuum degaser, an autosampler, a thermostated column compartment and a fluorescence detector. An Agilent Chemstation software package was used for instrument control, data acquisition and data handling. Separation was carried out on a Zorbax SB-aq C18 column (100 mm × 2.1 mm i.d., particle size 3.5 μm) from Agilent, coupled with a guard column with the same stationary phase (20 mm × 2.1 mm i.d, particle size 3.5 μm).

Plasma sample extractions were performed on Oasis® MCX 1 cc 30 mg extraction cartridges from Waters (Milford, MA, USA).

2.3. Chromatographic conditions

Chromatographic conditions were identical for plasma extracts and aqueous humor solutions. Mobile phase consisted of a mixture of acetonitrile and orthophosphoric acid 50 mM containing 5 mM sodium octane sulfonate, adjusted to pH 2.0 with sodium hydroxide 4N. Separation was performed under gradient conditions (Table 1), at a flow rate of 0.3 ml/min. Column temperature was fixed at 20 °C.

Fluorescence was measured with emission and excitation wavelengths set at 360 and 253 nm, respectively. Twenty microliters were injected onto the HPLC system.

Table 1
Optimised chromatographic conditions

Time (min)	Percentage phosphate buffer 50 mM with sodium octane sulfonic acid 5 mM, pH 2.0	Percentage acetonitrile
0	96	4
2	96	4
8	84	16
15	84	16
15.1	96	4

2.4. Standards and samples preparation

2.4.1. Standard solutions

Initial stock solutions of penciclovir and ganciclovir (2.5 mg/ml) were prepared by dissolving 2.5 mg of each compound in 1 ml of a mixture of methanol and water (50:50, v/v). Intermediate penciclovir standard solutions (250 and 10 μg/ml) in duplicate, and one intermediate ganciclovir standard solution (1250 μg/ml) were prepared from the primary stock solutions. Stock and intermediate solutions were kept frozen at −20 °C and remained stable for at least 12 months.

Working standard solutions (1, 2.5, 25, 50 and 100 μg/ml) were prepared in duplicate by appropriate dilution of each intermediate solution.

2.4.2. Plasma standard and quality control (QC)

Standard plasma and QC were prepared by spiking 450 μl of blank plasma with 50 μl of each working standard solution of penciclovir and 10 μl of I.S. solution, in order to obtain calibration standard samples of (0.25, 2.5, and 10 μg/ml) and QC of (0.1, 1 and 5 μg/ml). Plasma standards and QC were made in duplicate.

2.4.3. Aqueous humor standard and QC

For aqueous humor, no matrix effect was observed according to the low amount of protein content. Hence, calibration standard curve was made in water. Calibration standards (0.05, 0.1, 0.5, 1, 2, 5 μg/ml) were obtained in duplicate by appropriate dilution of the intermediate solution (10 μg/ml) in water. QC (0.05, 0.5 and 2 μg/ml) were prepared with blank aqueous humor. Considering the absence of sample preparation, no internal standard was added.

2.5. Samples extraction

Five hundred μl of plasma samples were spiked with 10 μl of I.S. and 500 μl of HCL 0.1N and vortexed vigorously for 30 s. The samples were loaded onto the Oasis® MCX column, and cartridges washed with 1 ml of hydrochloric acid 0.1N and 1 ml methanol. The cartridges were dried under vacuum. Compounds of interest were eluted with a mixture of methanol and ammonia solution 25% (95:5, v/v). After evaporation under a stream of nitrogen at 40 °C, residues were reconstituted in 500 μl of distilled water, and 20 μl were injected onto the HPLC system.

For aqueous humor samples, 20 μl were directly injected onto the HPLC system.

2.6. Method validation

The strategy applied for the validation of penciclovir in aqueous humor as well as in plasma, was based on the approach proposed by the "Société française des Sciences et Techniques Pharmaceutiques" (SFSTP) [44,45]. This procedure allowed us to confirm the linearity over the penciclovir tested concentration range, and to assess precision (repeatability and intermediate precision), trueness, and selectivity of the analytical method. To validate these different criteria, two kinds of plasma samples were prepared: calibration and validation samples corresponding to QC used in the routine analysis. The validation range was selected on the basis of the preliminary experiment to cover penciclovir expected concentrations in the clinical trial. These concentrations were between 0.05 and 5 μg/ml in the aqueous humor and between 0.1 and 10 μg/ml in the plasma. To better take into account between-day variability, a relative high number of days were considered for method validation (i.e. 8 days for plasma and 7 days for aqueous humour), as previously suggested by Hartmann et al. [46].

2.6.1. Selectivity

The selectivity was studied by analyzing six different sources of plasma and by injecting several concomitant medications taken by patients during the clinical study (aspirin, alprazolam, bromazepam, midazolam, atenolol, metoprolol, hydrochlorothiazide, amiodarone, amlodipine, nifedipine, simvastatine and acenocoumarol).

2.6.2. Linearity

The linearity of an analytical method is its ability within a definite range to obtain results directly proportional to the concentrations of the analyte in the sample [45]. A regression line was fitted on the back-calculated concentrations by applying the selected regression model. For plasma samples, three concentration levels ranging from 0.25 to 10 μg/ml were analysed in duplicate. In the case of aqueous humor samples, six concentration levels ranging from 0.05 to 5 μg/ml were analysed in duplicate.

2.6.3. Trueness

The trueness of an analytical procedure refers to the closeness of agreement between a conventionally accepted value and a mean experimental one [45]. Trueness was expressed as percentage recovery of the target value and assessed by means of validation standards in the matrix at three independent concentration levels (0.1, 1 and 5 μg/ml for plasma samples and 0.05, 0.5 and 2 μg/ml for aqueous humor). Samples were analyzed in duplicate for eight and seven days for plasma and aqueous humor, respectively.

2.6.4. Precision

The precision of an analytical procedure expresses the closeness of agreement between a series of measurements obtained from multiple sampling of the same homogeneous sample (independent essays) [45]. Precision was estimated by measuring repeatability and intermediate precision at the same concentration levels mentioned in Section 2.6.3. Variance of repeatability and intermediate precision were computed from estimated concentrations and precision was expressed by relative standard deviation (R.S.D.) at each level.

Table 2
Absolute recovery for plasma samples ($n = 3$) (%)

Days	Concentration (μg/ml)					
	0.1	0.25	1.0	2.5	5.0	10.0
1	88	91	94	88	94	93
2	98	89	94	96	98	77
3	86	81	86	92	85	94
4	98	89	94	96	98	77
5	82	83	90	83	87	88
6	78	71	71	103	100	96
Mean (± S.D.)	88.3 ± 8.2	84 ± 7.5	88.2 ± 9	93 ± 7	93.7 ± 6.3	87.5 ± 8.5

3. Results and discussion

3.1. Samples preparation

In order to separate penciclovir from endogenous purines, highly selective extraction and analytical methods have to be developed. Conventional liquid–liquid extraction (LLE) could not be used since penciclovir is very polar (log $P = -2.03$) [47]. Direct injection after proteins precipitation with either an organic solvent or an acid was described for penciclovir related compounds such as aciclovir and ganciclovir [14–27]. However, injection of strongly acid solutions reduces analytical column lifetime, and leads to numerous late-eluting peaks disturbing the analytical procedure [22]. Moreover, deproteinization with organic solvent such as acetonitrile requires an additional evaporation step or a dilution with water before separation on reversed-phase chromatography. Solid-phase extraction with apolar sorbent (C18) has already been used [28,32,33], but poor retention of hydrophilic compounds and lack of reproducibility was observed. Recently, a hydrophilic–lipophilic sorbent was successfully used for the extraction of nucleoside analogues [30,31].

In this paper, a solid-phase extraction method with an Oasis® MCX column, a polymeric mixed-mode cation-exchange and reversed-phase sorbent, was developed. Mixed-mode bonded phases are very selective for basic compounds [42] since the latter are selectively retained by hydrophobic and ion-exchange mechanisms [48]. Penciclovir and ganciclovir possess weak acid (pK_a 9.5 and 9.4) and weak base (pK_a 3.5 and 2.2) functional groups [25,47]. In HCl 0.1N, both compounds are positively charged and retained on the extraction support by a cation-exchange mechanism. The washing procedure was performed with an aqueous acidic solution to eliminate proteins, acidic and anionic compounds, while neutral and hydrophobic compounds were subsequently eliminated with methanol. Only basic compounds including penciclovir and ganciclovir were retained on the support with ionic interactions [48] and their elution was carried out with a methanolic solution at high pH. This method offers a simplified, highly selective and fast preparation procedure.

Under the selected extraction conditions, penciclovir showed mean absolute recoveries above 84% (Table 2). The recovery of the internal standard was also evaluated by comparing the mean peak areas of six extracted medium QC samples to peak area of reference solution of the same concentration. The mean recovery was above 70%.

3.2. Chromatography

Penciclovir analysis remains challenging since it is a nucleoside analogue and several endogenous substances can interfere [9]. Furthermore, penciclovir is a polar compound presenting weak interactions with reversed phase stationary phases. A chromatographic support (Zorbax SB-aq C_{18}) designed to retain hydrophilic and hydrophobic compounds in highly aqueous mobile phases, including 100% water, was firstly investigated without success. In order to enhance method selectivity and retention factor of penciclovir, an ion-pairing agent, sodium 1-octanesulfonate, was added to the mobile phase. In previous studies [14,24,28,30–33], ion-pairing agents (sodium octane-, heptane- and dodecylsulfonate) were often used at concentrations between 5 and 10 mM for nucleoside analogues. In our case, 5 mM sodium octanesulfonate allowed adequate retention of penciclovir and ganciclovir ($k' = 2.3$ and 5.9, respectively). Furthermore, with the selected stationary phase, it is possible to perform separations and enhance method sensitivity at low pH, without deterioration of column performances. Indeed, purines are not fluorescent in the non-ionised form [29] and fluorescence is heavily pH-dependent with best results obtained under strongly acidic conditions [29]. Several mobile phases were tested at different pH ranging from 2.0 to 4.0, and the best signal was obtained at pH 2.0 (Fig. 2). Moreover, at pH above 2.4, penciclovir peak was distorted with partial splitting (Fig. 2).

Temperature effect was also evaluated, and besides its effect on chromatographic parameters (selectivity, retention factor, etc.), it was found to be a critical parameter in term of sensitivity. Indeed, fluorescence increased with decreasing temperature (Fig. 3). However, column pressure increased at temperature below 20 °C and special equipment was necessary. Consequently, pH 2.0 and 20 °C were selected as a

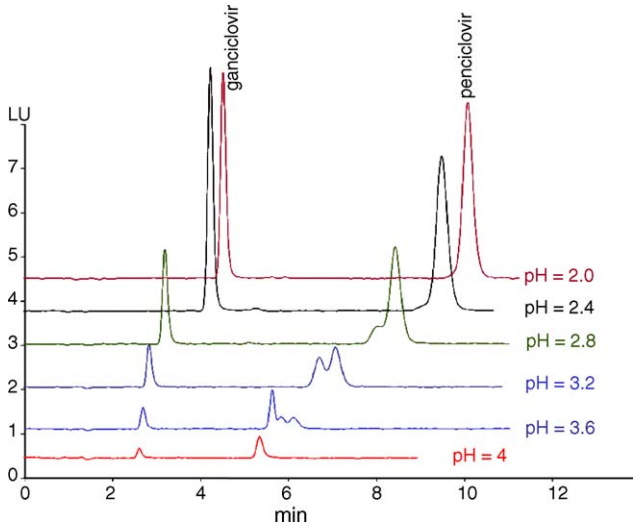

Fig. 2. Influence of buffer pH on penciclovir chromatographic behaviour. Chromatographic conditions: isocratic elution with a mixture of acetonitrile and orthophosphoric acid 50 mM containing 5 mM sodium 1-octanesulfonic acid (4:96, v/v) at a flow rate of 0.3 ml/min. pH was adjusted with sodium hydroxide 4N.

compromise between sensitivity, analytical column lifetime and analysis time.

3.3. Method validation

3.3.1. Selectivity

For both plasma and aqueous humor, selectivity was assessed by injecting several concomitant medications listed in the experimental part and no interference was observed. Furthermore, compounds structurally related to penciclovir (caffeine, theophylline, theobromine and methylxanthine) and six blank plasma and aqueous humor were also tested and, in all cases, no interference was observed.

3.3.2. Stability

The stability of penciclovir and I.S. in the final reconstituted extraction samples as well as in aqueous humor was assessed by keeping QC samples at room temperature and injecting after 24 h. The stability was also tested upon refrigeration (4 °C) for 1 month. The results were compared with those for QC samples freshly prepared. The results obtained were all comprised between 95 and 105% of the initial value. Consequently, no significant degradation of penciclovir and internal standard was observed.

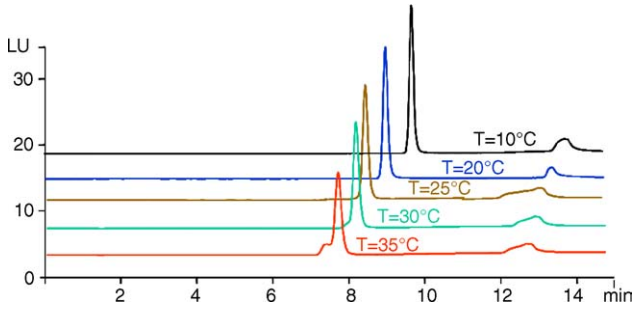

Fig. 3. Influence of temperature on penciclovir peak area and peak efficacy. Chromatographic conditions: See Section 2.3 and Table 1.

Table 3
Linearity data for penciclovir in plasma and aqueous humor

Calibration	Plasma (k = 8 days, n = 2)	Aqueous humor (k = 7 days, n = 2)
Intercept	0.00178	−0.01099
Slope	0.997	1.112
Correlation coefficient	0.996	0.997

3.3.3. Plasma samples

3.3.3.1. Calibration and linearity. For calibration, three concentration levels ranging from 0.25 to 10 μg/ml for penciclovir were selected. The relationship between peak area and concentration was linear in the calibration range considered. Since the hypothesis of variance homogeneity was rejected, the simplest unweighted regression model could not be retained. Therefore, a weighting factor of $1/x^2$ was chosen taking into account the relationship between natural variance logarithms and concentration as described elsewhere [49]. Further evaluations were conducted to check the model's adequacy such as residual plots examination and lack of fit test (data not shown). The regression equation is presented in Table 3.

3.3.3.2. Trueness and precision. For validation samples, three independent concentrations (0.1, 1 and 5 μg/ml) were analysed. The lowest concentration (0.1 μg/ml) was considered on the basis of a 10:1 signal to noise ratio, and analysed as independent samples to confirm the quantification limit (LOQ). Results are presented in Table 4 and demonstrated the method effectiveness for quantitative determination of penciclovir in plasma samples. Indeed, LOQ at 0.1 μg/ml was confirmed with an intermediate precision of about 20%, as recommended by regular guidelines [50]. Trueness, expressed as percentage recovery of the target value, exhibited excellent results with bias inferior to 3% throughout the tested range confirming the choice of the calibration

Table 4
Trueness, repeatability and intermediate precision for validation samples

Concentration (μg/ml)	Trueness (%)	Repeatability CVr (%)	Intermediate precision CVR (%)
Plasma (k = 8 days, n = 2)			
0.1	98.6	8.8	19.9
1	100.0	8.2	10.9
5	101.4	10.9	12.4
Aqueous humor (k = 7 days, n = 2)			
0.05	103.7	5.5	6.2
0.5	109.1	6.0	7.7
2	111.9	4.9	5.0

model. Method precision variability was inferior to 15% for the middle and highest concentration.

3.3.4. Aqueous humor

3.3.4.1. Calibration and linearity. According to the good selectivity towards endogenous components obtained by the developed method, no sample preparation was necessary and as previously described direct injection of samples was achieved.

For calibration, six concentration levels ranging from 0.05 to 5 μg/ml were analysed in duplicate. As previously observed with plasma analysis, a $1/x^2$ weighted linear regression was necessary to achieve good quantitative performances. After fitting the calibration curve each day, validation samples concentrations were computed from the analytical response obtained. The regression equation is presented in Table 3.

3.3.4.2. Trueness and precision. For validation samples, three independent concentrations (0.05, 0.5 and 2 μg/ml) were analysed in duplicate for seven days to assess method trueness and precision. Variance of repeatability and intermediate precision were computed from estimated concentrations and precision was expressed by relative standard deviation at each level. Trueness and precision results are presented in Table 4. For all tested concentrations, precision (R.S.D.) inferior to 15% was obtained. Trueness bias was inferior to 5% at the lowest tested concentration according to the appropriate choice of the weighting factor. At higher concentrations, slightly higher bias was observed, but still inferior to 15% of the theoretical value as recommended by FDA guidelines [50].

4. Application

The described method was successfully applied to a population pharmacokinetic study (approved by the Ethics Committee of Geneva University Hospitals) with human plasma and aqueous humor of patients undergoing a cataract surgery, after administration of a single 500 mg tablet of famciclovir (results will be published elsewhere). It permitted to monitor penciclovir in human plasma and aqueous humor for at least 12 h. In the tested population, concentrations ranged from 0.06 to 1.21 μg/ml in aqueous humor, and from 0.14 to 8.04 μg/ml in plasma. Typical chromatograms of plasma and aqueous humor samples of a patient that received a 500 mg famciclovir tablet are presented in Fig. 4A and B. A small peak is observed, with a retention time close to penciclovir. Since this peak is present only in the patients samples (aqueous humor and plasma) and possesses the same UV spectra, it probably corresponds to BRL42359, the desacetyl non-oxidized metabolite of famciclovir. Unfortunately, a standard solution of this metabolite was not available to confirm our assumption. Under optimal conditions, penciclovir was well separated from ganciclovir, with retention times of

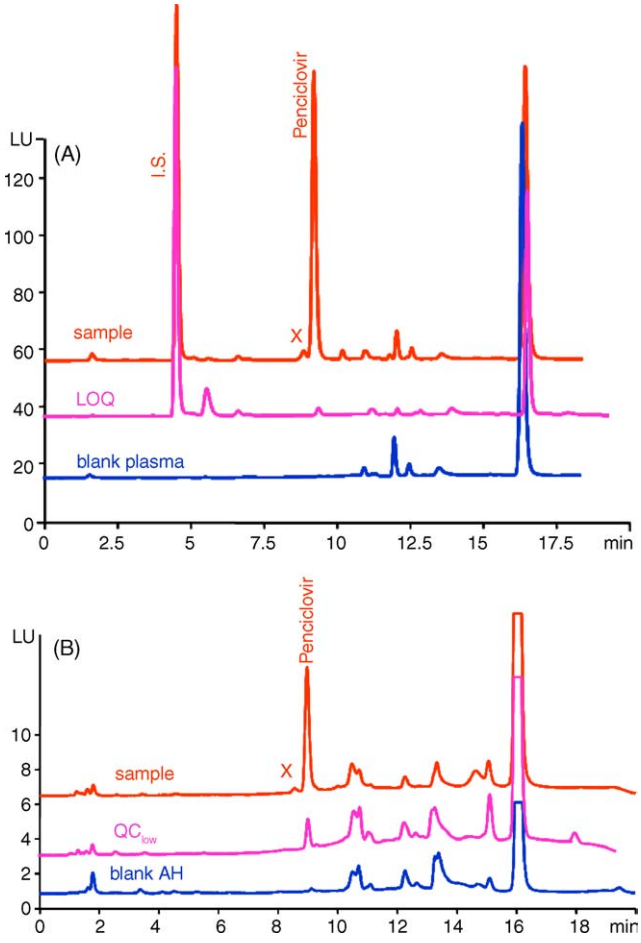

Fig. 4. Typical chromatograms of plasma (A) and aqueous humor (B), from one patient, 2 h after ingestion of a single famciclovir 500 mg tablet. Chromatographic conditions: See Section 2.3 and Table 1. X: unknown peak probably corresponding to BRL42359.

9.2 and 4.5 min, respectively, and no interfering peaks were observed.

5. Conclusion

A sensitive and reliable method was developed for penciclovir determination in human plasma and aqueous humour by liquid chromatography coupled to a fluorescence detector. A mobile phase constituted of phosphoric acid 50 mM at pH 2.0 provided the best penciclovir peak efficiency and shape, with respect to analysis time. An ion-pairing agent concentration of 5 mM was sufficient to separate penciclovir from endogenous purines and other polar interfering substances. Temperature and pH were found to be critical parameters in terms of sensitivity and efficiency and were fixed respectively at 20 °C and 2.0 as a compromise between sensitivity and column lifetime. For plasma samples, a solid-phase extraction method with an Oasis® MCX column was used, and good recoveries and high selectivity were obtained. Aqueous humor samples

were directly injected, owing to the low amount of protein content. Finally, the method was formally validated and successfully applied to the monitoring of penciclovir in plasma and aqueous humor in a pharmacokinetic study.

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