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Automated high-performance liquid chromatographic method for the determination of acyclovir in plasma☆

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Abstract

Owing to the low plasma concentrations of acyclovir obtained during pharmacokinetic studies after low-dosage oral administration, a sensitive, automated HPLC method had to be developed for determining acyclovir in a large number of plasma samples. Extraction and injection of the samples were done automatically by a Gilson ASPEC system using tC_{18}, 100-mg Sep-Pak Vac extraction columns. The extracts were chromatographed on a Nova-Pak C_{18} column with sodium octanesulphonate and methanol in the mobile phase. The analyte was detected at 250 nm. The calibration graphs were linear up to at least 1200 ng/ml and the limit of quantification was 10 ng/ml.

1. Introduction

Acyclovir {9-[(2-hydroxyethoxy)methyl]guanine}, an acyclic analogue of the natural nucleoside 2′-deoxyguanosine, selectively inhibits replication of members of herpes groups of DNA viruses, with low host cell toxicity [1]. Acyclovir is slowly and poorly absorbed from the gastrointestinal tract (20%) [2] and reaches its peak concentration in plasma *ca.* 1.5–2 h after an oral dose.

A number of radioimmunoassay [3–5] and HPLC [6–11] methods have been published for the determination of acyclovir in plasma. These methods did not meet our requirement, as we needed a sensitive and automated method to determine acyclovir in a large number of plasma samples for up to five half-lives of the drug after a single 400-mg oral administration to human volunteers. A sensitive assay procedure was required in order to obtain the necessary data for pharmacokinetic calculations. Published radioimmunoassay methods are sensitive enough but they require the proper antibody, which was not available to us. Of the published HPLC methods, only that of Mascher *et al.* [10] was sensitive enough, but it required step-gradient elution to wash the column after every injection and it was not automated.

This paper describes the first fully automated procedure for the determination of acyclovir in plasma using solid-phase extraction and UV detection.

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2. Experimental

2.1. Chemicals and reagents

All the reagents were of guaranteed analytical grade and were used as received. Sodium octanesulphonate (SOS) was obtained from Tokyo Kasei Kogyo (Tokyo, Japan). Acyclovir was 99.7% pure. Water was purified by passing it through a Millipore Milli-Q filtration system (18 MΩ cm resistivity) (Waters, Milford, MA, USA).

2.2. Analytical systems

A modular HPLC system was used which consisted of a pump (LC-6A; Shimadzu, Kyoto, Japan), a Waters Nova Pak C_{18}, 4-μm particle size, 150 mm × 3.9 mm I.D., stainless-steel cartridge (batch T12632) protected by an Upchurch (20 mm × 2 mm I.D.) precolumn, dry-packed with Perisorb RP-18 (30–40 μm) packing. A Shimadzu SPD-6A UV detector was used to measure the absorbance of the eluate at 250 nm. The chromatograms were recorded on an SP4290 integrator (Spectra-Physics, San Jose, CA, USA) and the data were sent via a LABNET network to a workstation with Winner for Windows software for automated data manipulation.

Sample processing and injections were done by an ASPEC system (automated sample preparation with extraction columns) from Gilson (Villiers le Bel, France) utilizing Waters Sep-Pak Vac trifunctional C_{18} (tC_{18}) (100 mg; 1 ml) extraction columns.

2.3. Chromatography

The mobile phase consisted of methanol–Na_2HPO_4 (0.01 *M*) containing 0.01 *M* sodium octanesulphonate (7:93, v/v), with the final apparent pH of the mobile phase adjusted to pH 2.80 with concentrated orthophosphoric acid, pumped at a flow-rate of 1 ml/min, and the column temperature was maintained at 40°C. The retention time of acyclovir was *ca.* 3.6 min.

2.4. Sample preparation

Plasma standards and quality controls. An accurately weighed amount of acyclovir was dissolved in water and an aliquot was added to drug-free plasma to obtain a stock standard solution containing *ca.* 1.2 μg of acyclovir per millilitre of plasma. Subsequent dilutions with plasma were made to obtain eight adequate working standard solutions covering the expected range. For quality controls a stock standard solution in water was prepared and different amounts were individually added to three different volumes of drug-free plasma to obtain quality controls with concentrations $C_{max} = 601$ ng/ml, $C_{av} = 301$ ng/ml and $C_{min} = 88$ ng/ml. These standards and quality controls were divided into aliquots and stored at −20°C under the same conditions as the actual trial samples. With each batch of samples analysed each day a set of standards and quality controls were processed and were scattered among the 68 samples of each batch.

Plasma samples (0.5 ml) were transferred to 5-ml glass tubes and mixed with 0.5 ml of 5 m*M* sodium octanesulphonate (adjusted to pH 2.85 with concentrated orthophosphoric acid). The samples were briefly vortex mixed to achieve thorough mixing and placed in the ASPEC system.

The apparatus was programmed to condition each Sep-Pak Vac extraction column with 1 ml of methanol, followed by 1 ml of 5 m*M* sodium octanesulphonate (pH 2.85), just before use. The plasma mixture was loaded on to the column and washed with 0.5 ml of 5 m*M* sodium octanesulphonate (pH 2.85). The analytes were eluted with 300 μl of 5 m*M* sodium octanesulphonate (adjusted to pH 8.50 with 4 *M* NaOH)–methanol (4:1) into a clean tube and mixed by bubbling air and a 130-μl aliquot was automatically injected on to the HPLC column.

Validation. During the validation of the analytical method, standards and quality controls were prepared in plasma over the range of concentrations estimated to be $2 \cdot C_{max}$ expected (1244 ng/ml) down to $1/8 \cdot C_{min}$ expected (2.71

ng/ml). The controls were analysed five times to determine the accuracy and precision of the procedure. As the lower concentration controls often fall in a range close to the limit of detection, these serve as a measure of the limit of quantification (LQ). The limit of quantification was chosen as that concentration at which the analyte could still be determined with acceptable precision and accuracy (maximum R.S.D. = 15%). The LQ was set at 10 ng/ml with an R.S.D. of 5% and an accuracy of 97% with a signal-to-noise ratio of 7. The specificity of the assay with respect to related endogenous compounds was tested by analysing samples from six different volunteers during the method development and from 24 different volunteers during the study. No interfering peaks from endogenous plasma compounds were found during chromatography of these samples. As this was a pharmacokinetic study performed with volunteers, the assay procedure was not tested against possible interferences by concomitantly ingested drugs.

The absolute recovery was tested at concentrations of 601, 301 and 88 ng/ml by comparison with directly injected aqueous standard solutions. The recoveries were 87%, 82% and 88%, respectively.

3. Results and discussion

3.1. Selection of extraction method

From a number of experiments involving plasma precipitation with perchloric acid, trifluoracetic acid, acetonitrile and heavy metals, it was apparent that we would not obtain the necessary sensitivity required to measure acyclovir in plasma for five half-lives of the drug after a single 400-mg oral dose unless the samples could be prepurified and concentrated. Acyclovir is very hydrophilic and therefore it is very difficult to extract it from plasma using organic solvents. We tried to extract it from plasma at various pH levels (1–12) using from very non-polar solvents such as hexane to more polar solvents such as methylene chloride, without success. Ion-pairing extractions using di(2-ethylhexyl)phosphoric acid in chloroform as an ion-pairing reagent also proved unsuccessful. Solid-phase extraction columns tested unsuccessfully included silica, aminopropyl and non-polar phases such as C_2, C_8 and C_{18}, using buffers at various pH values. The retention of acyclovir on the benzenesulphonylpropyl ion-exchange cartridge was acceptable when the acyclovir was dissolved in water but not in plasma, even after plasma precipitation before loading of the sample on to the SPE column.

Good retention of acyclovir on a Novapak C_{18} analytical column was obtained when the mobile phase contained 5 m*M* sodium octanesulphonate at pH 2.8. This prompted us to investigate whether we could achieve a similar retention of acyclovir on a C_{18} SPE column using the same SOS solution. The optimum conditions for retention of acyclovir on a Bond-Elut C_{18} column (1 ml, 100 mg; Varian) were obtained when the plasma was mixed with an equal volume of 5 m*M* sodium octanesulphonate at pH 2.8 and the extraction column prepared with the SOS solution. At any pH value of the SOS solution below or above 2.8, a marked decrease in the retention of acyclovir on the SPE column was found. The recovery of acyclovir from C_{18} extraction columns from different manufacturers and from the same manufacturer with different batch numbers varied considerably (Table 1). With the Waters tC_{18} columns we obtained the best recoveries and batch-to-batch variation.

Elution of acyclovir from the extraction column can easily be achieved with organic solvents such as methanol, but direct injection of the eluate on to the analytical column results in peak broadening due to the low concentration of organic modifiers in the mobile phase. A solution containing 20% methanol in 5 m*M* sodium octanesulphonate (pH 8.5) gave 100% elution of acyclovir from the extraction column and did not compromise the peak shape after direct injection on to the analytical column. Higher concentrations of methanol caused severe peak-shape deterioration.

TABLE I

Differences in recovery of acyclovir from different C_{18} solid-phase extraction (SPE) columns.

SPE column	Lot No.	Recovery (%)
Supelclean LC_{18} (Supelco)	SP0386	45
Extra Sep C_{18} (Lida)	1253	51
Clean-UP C_{18} (World Wide Monitoring)	Test sample	59
Sep-Pak Vac tC_{18} (Waters)	P0334A1	85
	P0342A2	79
Bond Elut C_{18} (Varian)	071365	45
	070695	24
	070975	57

Fig. 1 shows representative chromatograms obtained from plasma analyses and demonstrates the lack of interfering endogenous compounds in the blank plasmas. Quantification was achieved using the peak-height values of acyclovir. The calibration graphs constructed with eight standard concentrations were linear over a wide range (5–1200 ng/ml) and almost passed through the origin [$y = -69.348 + 29.793x$ correlation coefficient $r^2 = 0.999$; y = peak height (arbitrary units) and x = concentration (ng/ml)]. The within- and between-day precision of the method is indicated in Table 2.

Although previous workers claimed better sensitivity for the detection of acyclovir using fluorescence detection, we decided to use UV detection as it avoided the need to work at very low pH values or low temperatures, as is required with fluorescence detection to obtain adequate sensitivity. Using an automated preconcentration step and UV detection, our method was sensitive enough for use in pharmacokinetic studies. The extraction and chromatographic procedures are fully automated and take only 10 min from injection of a sample to extraction and injection of a second sample. Minimum sample handling is required and analyses can be done 24 h per day.

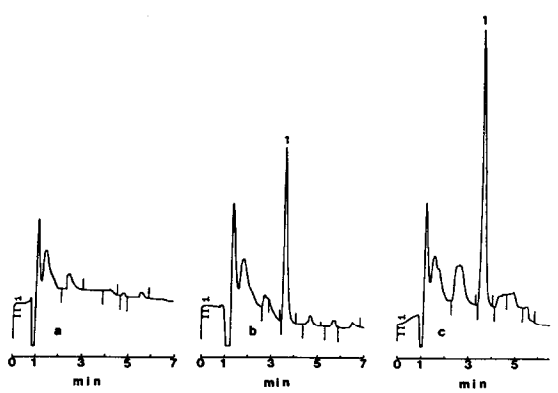

Fig. 1. Representative chromatograms obtained showing extractions from (a) blank plasma, (b) a trial sample equivalent to 567 ng/ml and (c) a plasma standard equivalent to 863 ng/ml. For HPLC conditions, see text.

3.2. Factors affecting chromatographic resolution

Smith and Walker [9] described the relationship between pH, an ion-pair reagent, sodium heptanesulphonate and the k' values for acyclovir. From that it was clear that appreciable retention of acyclovir occurred once the eluent pH decreased below 3.2. An acidic mobile phase containing sodium octanesulphonate was chosen as it permitted better retention of the compound on the reversed-phase column. Slight changes in pH significantly affected the retention of acyclovir. It was found that the pH of the mobile phase had to be lowered slightly after 200–300 injections in order to obtain reasonable retention times and optimum resolution from co-extracted endogenous components. Smith and Walker [9] used a polystyrened–divinylbenzene stationary phase to obtain better column stability, but large relative standard deviations were found below 1.2 μg/ml owing to the low column efficiencies for acyclovir.

Although the pH of the mobile phase eventually approached the commonly suggested limit (pH 2) for use with silica-based analytical columns, reasonable column lifetimes were observed. The columns were replaced after about 1000–1500 injections as lowering of the pH no longer resulted in separation of the analyte from the endogenous compounds. These columns were later tested and found to be still useful in the chromatography of other compounds. The analytical column appears to be modified by the

TABLE II

Within- and between-day precision of the method

Within-day ($n = 5$)			Between-day ($n = 20$–24)		
Nominal plasma concentration (ng/ml)	Nominal/found (mean) (%)	R.S.D. (%)	Nominal plasma concentration (ng/ml)	Nominal/found (mean) (%)	R.S.D. (%)
1244	93	7.2	1006	96	7.2
623	95	7.8	457	99	9.3
312	92	3.7	31.8	105	8.4
155	98	4.6			
77.3	98	7.2			
38.2	101	2.2			
20.4	109	2.3			
10.1	103	5.1			
5.44	106	10			

injection of the plasma extracts, which later renders them useless for further determinations of acyclovir but not for other compounds; the guard-column packing was changed every day to protect the analytical column from extracted plasma residues.

4. Conclusions

The method is sensitive enough to determine concentrations of acyclovir in low plasma volumes for 24 hs after a single 400-mg oral dose. Analyses can be done 24 h per day as the extraction, injection, chromatographic and data manipulation steps are completely automated.

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