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SIMPLE HIGH-PERFORMANCE LIQUID CHROMATOGRAPHIC METHOD FOR THE ANALYSIS OF 9-(2-HYDROXYETHOXYMETHYL)GUANINE (ACYCLOVIR) IN HUMAN PLASMA AND URINE

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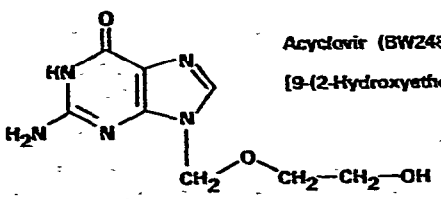
SUMMARY

Acyclovir, a new antiviral drug for human use, could not be solvent extracted and was retained poorly by reversed-phase (RP) columns. Drug in urine and plasma (deproteinised) could be chromatographed successfully as an ion-pair (with heptanesulphonic acid) on RP-18 columns. The validated method had the required sensitivity for a wide variety of clinical situations including metabolic and pharmacokinetic studies.

INTRODUCTION

Acyclovir [9-(2-hydroxyethoxymethyl)guanine] is a modified nucleoside which shows strong activity against viruses of the Herpes group [1]. Methods, other than microbiological, for the analysis of acyclovir have been based on radioimmunoassay (RIA) [2] or high-performance liquid chromatography (HPLC) utilising ion-exchange [1, 3] or reversed-phase columns [2]. Following



Acyclovir (BW248U)

[9-(2-Hydroxyethoxymethyl)-guanine]

administration of acyclovir to man it was found that the published HPLC methods lacked selectivity and sensitivity for the analysis of the drug in plasma and urine. Although RIA had the relevant sensitivity we encountered occasional problems in analysing plasma and urine from patients because of possible cross-reactivity with the antiserum. Continued development of RIA

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may overcome this problem. As an alternative we looked at ways of improving the HPLC methods.

Selective extraction of acyclovir from plasma or urine was unsuccessful because of its poor lipid solubility. Attempts to couple counter-ions of good lipid solubility followed by extraction also failed. However, reversed-phase ion-pair HPLC met with success and proved superior to either reversed-phase or ion-exchange techniques for the measurement of unchanged drug. This method is reported below.

METHODS

Chemicals and reagents

Acyclovir was obtained from Wellcome Foundation (Dartford, Great Britain); heptanesulphonic acid, sodium salt, HPLC grade from Fisons (Loughborough, Great Britain); and sodium acetate, barium hydroxide and aluminium sulphate, AnalaR grade from BDH (Poole, Great Britain). All aqueous solutions were made up using deionised water (Deioniser type C810, Elgastat, High Wycombe, Great Britain).

Glassware

Centrifuge tubes, 10 ml graduated were purchased from M.S.E. (Crawley, Great Britain), and microvials with PTFE-lined caps from Waters Assoc. (Stockport, Great Britain).

Instrumentation

The chromatography was carried out on a Model SP8000 high-performance liquid chromatograph system using a SP8310 fixed-wavelength (254-nm) detector (Spectra-Physics, St. Albans, Great Britain). An autosampler (WISP-710A, Waters Assoc.) was also used. A refrigerated centrifuge (Mistral 2L, M.S.E.) fitted with an eight-place angle head was used to remove the suspended solids and precipitated proteins from the biological samples.

Determination of acyclovir in plasma

Standard solutions of acyclovir ranging from 0—50 μM were prepared by dilution of an aqueous stock solution (1 mM) with heparinised human plasma. An aliquot of standard or plasma (0.5 ml) was placed in a 10-ml centrifuge tube followed by 200 μl of a 5% w/v aluminium sulphate solution. The tube contents were vigorously mixed (Whirlimixer, Fisons) and a 400-μl aliquot of 0.15 M barium hydroxide was added followed by further mixing. The suspended solids were forced into a sediment by centrifugation (5000 g for 20 min at 10°C). The tubes were covered (Parafilm, American Can Company, Greenwich, CN, U.S.A.) and left to stand overnight at 4°C to aid precipitation. After uncovering, the tubes were again centrifuged (5000 g for 20 min at 10°C) and the clear supernatant was carefully transferred to a microvial which was capped in preparation for injection using the automatic sampler. Standards and samples were analysed in duplicate. The protein precipitation method was based on that of Gyure [4] which was shown to give good recovery of purines. The recovery of acyclovir by this method was shown to be greater than 90%.

Radioimmunoassay of acyclovir in plasma

The method of Quinn et al. [2] was used.

Determination of acyclovir in urine

Standard solutions of acyclovir with concentrations ranging from 0—500 μM were prepared by dilution of aqueous stock solution (1 mM) with filtered human urine. Samples were loaded into the autosampler and injected directly onto the liquid chromatograph. Because of the wide operating range of the urine assay dilution of unknown urine samples was rarely necessary.

HPLC conditions

A 250 mm × 4.6 mm I.D. column packed with 5-μm ODS-silica was used (e.g. Zorbax ODS, 4.6 μm; Du Pont, Hitchin, Great Britain). The mobile phase was an aqueous solution of 0.005 M sodium acetate and 0.0025 M heptanesulphonic acid, sodium salt, filtered before use through grade 50 filter paper (Whatman). The final pH was 6.5. Table I gives the optimum conditions for the HPLC of acyclovir in plasma and urine.

TABLE I

OPTIMUM CONDITIONS FOR HPLC OF ACYCLOVIR IN PLASMA AND URINE

The fixed-wavelength (254-nm) detector was operated at 0.005 a.u.f.s.

	Injection size (μl)	Mobile phase flow-rate (ml/min)	Column temperature (°C)	Minimum run time (min)
Plasma	60	1.2	50	15
Urine	15	1.5	35	35

RESULTS

In plasma extracts acyclovir was well resolved from the void volume and other endogenous peaks (see Fig. 1). No interference was found from the major acyclovir metabolite (9-carboxymethoxymethylguanine), guanine, thioguanine and related purines. In urine a co-eluting endogenous compound was seen in some subjects and made quantitation below 10 μM difficult in these subjects. This effect diminished at higher acyclovir concentrations. A long run time was necessary for urine extracts because of late eluting compounds present in urine under these conditions (see Fig. 2). An increase in the acyclovir peak area, and therefore sensitivity was achieved by larger injections. A plot of peak area (y) obtained at 0.005 a.u.f.s. in μV/sec, against the injection volume of a 10 μM solution of acyclovir (x) gave a linear calibration curve of the form; $y = 1324.8x - 7181$ ($r > 0.99$, $n = 11$ over 11 values of x). It was possible to inject 160 μl of a 10 μM acyclovir solution without affecting peak shape. Shorter run times were possible by using higher column temperatures. However, above 50°C for plasma and 35°C for urine, other endogenous peaks became unresolved from acyclovir making integration of peak areas difficult especially

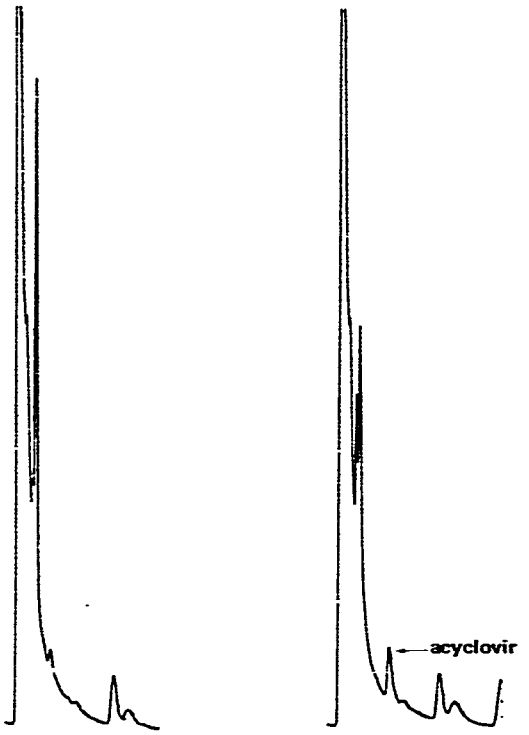

Fig. 1. Typical chromatograms from the plasma of a patient before and after receiving intravenous acyclovir. (a) Blank plasma; (b) plasma containing approximately 7 μM acyclovir.

at low concentrations. Under the described conditions acyclovir had retention times of about 364 and 1360 sec in plasma and urine, respectively.

Plasma validation

The HPLC method showed a linear response in plasma over the range 0—50 μM. A plot of peak height in mm (y) against acyclovir concentration in μM (x) gave a linear calibration curve of the form, $y = 1.9827x + 0.774$ ($r>0.99$, $n = 48$ over 8 values of x). Similar calibration curves were obtained whether peak height or peak area was used on the ordinate. Better precision was apparent at low acyclovir concentrations using peak height (Table II). However, when the percentage standard deviations from the peak height and peak area measurements were averaged, then compared by the t-test modified for inequality of variance, no significant difference was seen at the 5% level. No advantage on precision was obtained after adding an internal standard (oxipurinol). An unkown acyclovir peak height or area from the HPLC chromatogram was quantitated simply by reference to a standard calibration curve.

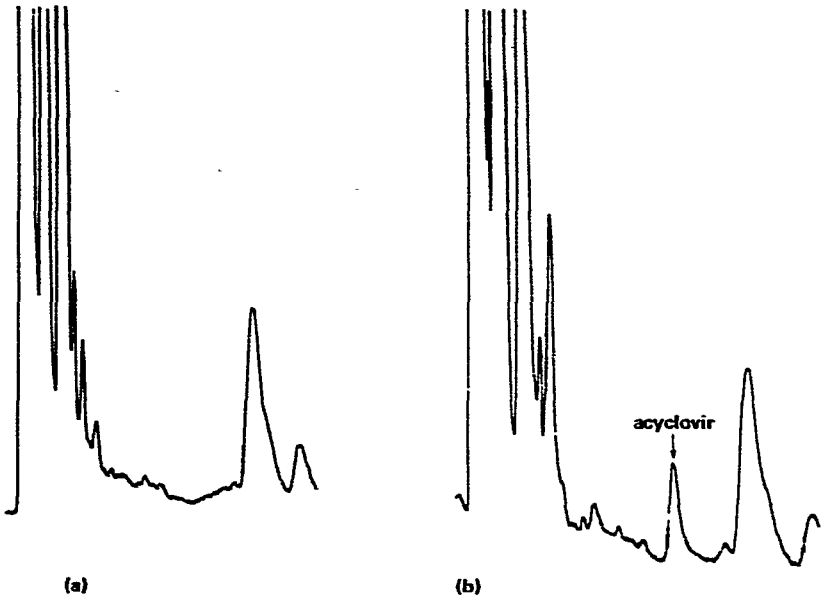

Fig. 2. Typical chromatograms of (a) blank urine and (b) urine containing 20 μM acyclovir.

TABLE II

COMPARISON OF THE ERRORS INVOLVED WHEN PEAK HEIGHT OR PEAK AREA WAS USED IN MEASURING ACYCLOVIR CONCENTRATIONS IN HUMAN PLASMA

Each observation was the mean of six determinations.

Concentration of acyclovir (μM)	Peak height (mm)	S.D. (%)	Peak area (μV/sec)	S.D. (%)
0	0	—	0	—
1	2.25	18.6	19,687	32.1
2	4.58	12.8	40,705	24.6
5	10.75	17.0	283,595	15.0
10	21.5	13.0	464,492	9.2
15	32.4	4.7	680,794	6.9
25	49.2	4.3	972,110	6.4
50	99.75	6.5	2,070,830	5.0

Urine validation

The HPLC method showed a linear response over the range 0—500 μM. However, when undiluted urine from some patients was injected an unknown co-eluting peak was seen on the HPLC chromatogram. This raised the lower reliable detection limit to 10 μM but if the peak was missing, quantitation to 1 μM was possible. A plot of peak height in mm (y) against acyclovir concentration in μM (x) gave a linear calibration curve of the form, $y = 0.15626x + 2.304$ ($r > 0.99$, $n = 32$ over 8 values of x). As in the plasma validation no internal standard was needed and peak height or area could be used.

TABLE III

COMPARISON OF HPLC AND RIA FOR THE DETERMINATION OF ACYCLOVIR IN HUMAN PLASMA

In the RIA all samples above 15 μM were diluted 1:10 with the same human plasma as used for the zero so that only the most precise part of the calibration curve (1—10 μM) was used. No dilution was necessary for the HPLC method.

Actual concn. acyclovir (μM)	Concn. acyclovir by HPLC* (μM)			Mean (± S.D.)	Concn. acyclovir by RIA** (μM)			Mean (± S.D.)
0	0,	0,	0,	0	0,	0,	0,	0
	0,	0,	0		0,	0,	0	
2.8	2.605,	1.955,	2.12,	2.65	2.72,	2.52,	2.89,	2.705
	2.995,	3.42,	2.81	(0.55)	2.75,	2.65,	2.70	(0.12)
5.6	4.83,	4.30,	3.825,	4.92	5.03,	5.18,	5.19,	5.35
	4.37,	5.91,	6.26	(0.97)	5.70,	5.63,	5.39	(0.268)
14.0	12.63,	14.44,	13.535,	13.92	13.6,	13.4,	13.4,	13.53
	13.215,	14.29,	15.385	(0.99)	14.4,	13.1,	13.3	(0.45)
25.9	23.455,	23.71,	25.43,	23.90	24.1,	23.9,	25.9,	25.67
	23.49,	25.2,	28.29	(1.23)	28.6,	26.7,	24.8	(1.79)
42.0	38.945,	41.065,	41.24,	42.67	41.63,	41.3,	41.3,	41.37
	45.94,	42.68,	46.125	(2.89)	41.3,	42.5,	40.2	(0.74)
70.0	67.165,	64.29,	70.39,	71.95	69.0,	64.0,	74.0,	70.58
	72.86,	74.445,	82.56	(6.38)	77.0,	69.8,	69.7	(4.47)

*Mean of duplicates.
**Mean of triplicates.

Correlation of HPLC and RIA methods for plasma acyclovir

A set of seven standards was set up in six replicates over the range 0—70 μM acyclovir using plasma which was known not to cross-react in the RIA method. The standards were then analysed blind by both HPLC and RIA. At concentrations greater than 15 μM samples were diluted 1:10 with blank human plasma before RIA so that only the most precise part of the calibration curve was used. On completion the code was broken and the answers were subjected to statistical analysis. A good correlation was obtained ($r>0.99$, $n = 42$; see Table III). At low concentration the RIA had better precision than the HPLC method.

DISCUSSION

Using conventional reversed-phase columns acyclovir was poorly retained and only new, high-efficiency columns had sufficient resolution to allow quantitation. Ion-exchange columns could resolve acyclovir but the poor column efficiency and band broadening effects limited this method to high concentrations of acyclovir. The described ion-pair method combined the advantages of both high resolution and long column life from the previous methods to allow reliable quantitation of acyclovir down to 1 μM in plasma and 10 μM in urine. Greater sensitivity was possible with high-efficiency

columns but the absolute sensitivity was a function of the detector response and the molar extinction coefficient of acyclovir (13,600 at λ_{max} 250 nm). For most situations adequate sensitivity was achieved using the described conditions. The stability and low noise levels of the fixed-wavelength (254-nm) detector outweighed any advantages of using a variable-wavelength detector at λ_{max}. Including automatic injection, 90 samples of plasma or 40 samples of urine can be analysed each day by one technician. Normally a 0.5-ml sample of plasma was needed per determination, however this could be reduced if necessary. Urine can be injected directly into the HPLC so only a few microliters of sample are needed and a result can be available within 35 min from receipt of the sample.

RIA in general has high sensitivity but can suffer from problems of cross-reactivity and non-linear calibration curves. Fortunately the published RIA method currently utilises a fairly specific antibody, the only known major cross-reaction occurring with thioguanine. Minor cross-reactivity does occur but can be overcome either by allowing for the percentage of zero binding of the pre-drug sample or by setting up standards in the pre-drug sample. Where this was not possible the RIA had a tendency to measure apparent acyclovir in some specimens from people who had not yet received the drug. This effect was most noticeable in patients who were immunosuppressed with the drug Azathioprine (Imuran). Although this effect was insignificant at low acyclovir concentration large errors could occur at high concentration by virtue of the logarithmic nature of the calibration curve used. In the RIA, some problems were also experienced in getting a good precipitate after the addition of ammonium sulphate when the original plasma sample contained large amounts of fat. In the described HPLC method no acyclovir was detected in any plasma sample from subjects who had not received the drug, making the method more universally applicable. The HPLC method had a linear calibration curve and no internal standard was needed. Therefore relatively few standards need be run for comparable accuracy with RIA. Also the accurate linear dynamic range of the HPLC method is wider than the described RIA method which obviates the need for preliminary range finding. The HPLC and RIA methods were found to correlate well under optimum conditions and were both useful alternatives. In summary, the HPLC method described has sufficient sensitivity and reliability for the measurement of acyclovir in a wide variety of clinical situations and can also be used for pharmacokinetic and metabolism studies [5, 6].

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