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Short communication

Determination of acyclovir and its metabolite 9-carboxymethoxymethylguanine in serum and urine using solid-phase extraction and high-performance liquid chromatography

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Abstract

A reversed-phase ion-pair high-performance liquid chromatography method for the determination of acyclovir and its metabolite 9-carboxymethoxymethylguanine is described. The samples are purified by reversed-phase solid-phase extraction. The components are separated on a C_{18} column with a mobile phase containing 18% acetonitrile, 5 m*M* dodecyl sulphate and 30 m*M* phosphate buffer, pH 2.1, and measured by fluorescence detection using an excitation wavelength of 285 nm and an emission wavelength of 380 nm. Detection limits are 0.12 μ*M* (plasma) and 0.60 μ*M* (urine) for acyclovir, and 0.26 μ*M* (plasma) and 1.3 μ*M* (urine) for metabolite. Correlation coefficients that were better than 0.998 were obtained normally. This analytical method, which enables simultaneous measurement of parent compound and metabolite, has been used in kinetics studies and for therapeutic drug monitoring in different patient groups with variable degrees of renal dysfunction.

Keywords: Acyclovir; 9-Carboxymethoxymethylguanine

1. Introduction

Acyclovir (ACV), an acyclic analogue of 2-deoxyguanosine, has highly selective biological activity, which results in the inhibition of herpes virus replication. It has been shown to be effective in the treatment of serious viral infections with both *Herpes simplex* and *Varicella zoster*. It has also been used in low doses as a prophylactic against cytomegalovirus (CMV) infections in connection with immunosuppressive therapy in organ recipients [1]. After more than a decade of therapeutic use of ACV, it has been shown that it is well tolerated in a wide variety of disease states, population types and age groups. However, recent reports of possible neurotoxic effects of ACV in patients with renal disease has renewed interest in identifying reasons behind the inter-patient variations in the kinetics of the drug [2]. ACV is mainly eliminated unchanged in urine. Kinetic studies in healthy volunteers have shown wide variations in renal clearance and total urinary recovery. About 30–70% of the dose is recovered unchanged in the urine. The incomplete urinary recovery of ACV could be attributed, in part, to the formation of 9-carboxymethoxymethylguanine

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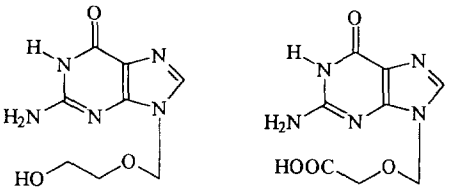

Fig. 1. Structure of acyclovir and its main metabolite.

(CMMG) [3]. The structures of ACV and CMMG are shown in Fig. 1.

Several high-performance liquid chromatography (HPLC) methods [4–11] have been published for the determination of ACV. One of those studies [7] co-analyzed CMMG. The chromatography time in this case was more than 40 min for each injection.

We present here a simple, fast and sensitive HPLC method for the determination of ACV and CMMG in serum and urine. It can be used for pharmacokinetic studies in patients.

2. Experimental

2.1. Materials

ACV and CMMG were gifts from Wellcome Foundation (Dartford, UK). Sep-Pak Light C_{18} cartridges were obtained from Waters (Milford, MA, USA). 1-Dodecyl sulphate (sodium salt) was a reagent of electrophoresis purity (Bio-Rad, Richmond, CA, USA). Acetonitrile was of HPLC grade. All other chemicals were analytical reagents. The water used was deionized.

2.2. Apparatus

The chromatographic equipment consisted of a 2150 pump (Pharmacia LKB, Sollentuna, Sweden), a 7125 injector (Rheodyne, Berkeley, CA, USA) with a 20-μl loop, an Ultrasphere ODS 75×4.6 mm I.D. reversed-phase column (3 μm particles; Beckman, Berkeley, CA, USA) and an RF-530 fluorescence detector (Shimadzu, Tokyo, Japan), with fixed band widths (excitation: 18 nm; emmission: 22 nm) and a fixed time constant (1.5 s).

2.3. Chromatographic conditions

The eluent was a 30 m*M* phosphate buffer, pH 2.1, containing 5 m*M* dodecyl sulphate and 18% acetonitrile. A 4.08-g amount of potassium dihydrogen phosphate, 1.45 g of sodium dodecylsulphate and 15 ml of 3.85 m*M* phosphoric acid were disolved in about 800 ml of water. A 180-ml volume of acetonitrile was added and the volume was brought to 1 l with water. The flow-rate was 1.5 ml/min and the temperature was ambient. The excitation wavelength was set at 285 nm and the emission wavelength was set at 380 nm.

2.4. Sample preparation

2.4.1. Serum

A 500-μl volume of serum was mixed with 500 μl of a saturated sodium chloride solution in water. The mixture was pushed through a Sep-Pak Light C_{18} cartridge (pretreated with 1 ml of methanol and 1 ml of water) with a plastic syringe. The flow-rate through the Sep-Pak cartridge was about 25 μl/s in this and subsequent steps. The cartridge was washed with 500 μl of a 50% saturated sodium chloride solution in water. ACV and CMMG were eluted with 1000 μl of a 3% acetonitrile solution in 38 m*M* phosphoric acid. The last 750 μl of this eluate were collected and 20 μl were injected into the column.

2.4.2. Urine

A 100-μl volume of urine was mixed with 900 μl of 50% saturated sodium chloride solution in water. The treatment on the Sep-Pak cartridge was thereafter the same as for serum (Section 2.4.1).

3. Results and discussion

Calibration curves were obtained by analysis of serum and urine samples that were spiked with ACV and CMMG. A linear relationship between height and concentration was observed up to 64 μ*M* for ACV and up to 80 μ*M* for CMMG in serum. Corresponding values in urine were 800 and 540 μ*M* for ACV and CMMG, respectively. Coefficients of variation at different concentrations in serum are shown in Table 1. In routine analysis, correlation

Table 1

Compound	Concentration (μM)	C.V. (%)
ACV	0.46	3.1
ACV	7.07	1.4
ACV	27.7	2.4
CMMG	0.88	8.3
CMMG	5.40	3.4
CMMG	27.0	4.3

$n=6$.

coefficients that were better than 0.998 were obtained normally for ACV in serum. The calibration concentrations were 0, 0.5, 1.0, 2.0, 4.0, 8.0 and 16.0 μM. A quality control sample spiked with 6.60 μM of ACV was determined to be 6.45 μM, with a coefficient of variation of 5.7% (twelve days). The minimum detectable concentrations (three times the background noise) were 0.12 μM of ACV in serum and 0.60 μM in urine. The corresponding figures for CMMG were 0.26 and 1.3 μM, respectively. Chromatograms of blank serum, serum spiked with ACV and serum spiked with CMMG are shown in Fig. 2. Chromatograms of serum and urine from kidney transplant recipients are shown in Fig. 3.

As ACV is a weak acid, with a pK_a of 9.25, and a weak base, with a pK_a of 2.27 [12], it is possible to chromatograph it as an ion-pair with a strong acid as the counter-ion, if the pH is under or near the latter pK_a. Dodecyl sulphate gave an ion-pair with very good retention. Protein precipitation with 0.4 M trichloric acid (1:1, v/v) and UV detection at 255 nm produced chromatograms with too many interfering peaks. Fluorescence detection as used by Salamoun

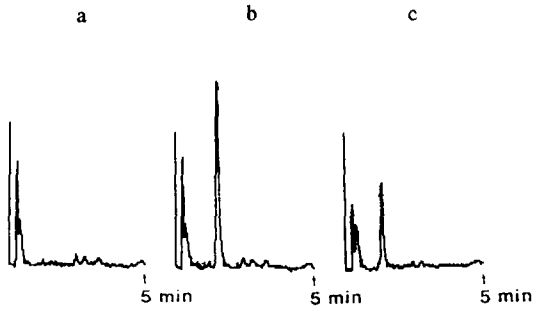

Fig. 2. Chromatograms of blank serum (a) and of serum spiked with 2 μmol/l ACV (b) and 2 μmol/l CMMG (c). Recorder attenuation=2.

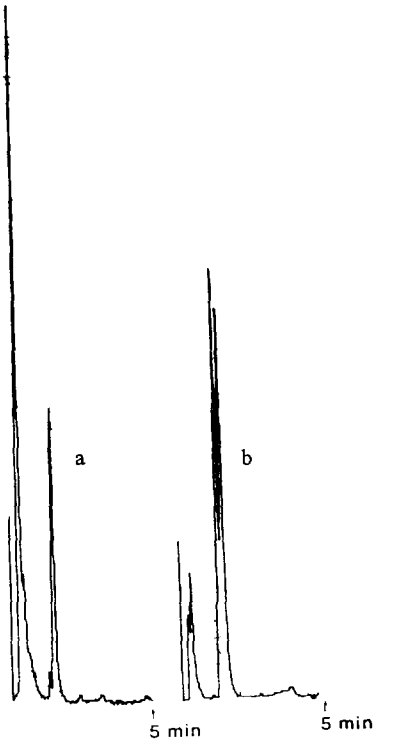

Fig. 3. Chromatograms of serum (a) and of urine (b) from kidney transplant recipients. The concentrations are 3.1 μM of ACV and 0.7 μM of CMMG in serum (recorder attenuation=2); 168 μM of ACV and 396 μM of CMMG in urine. Recorder attenuation=16.

et al. [6] gave cleaner chromatograms, but still there were late-eluting peaks disturbing the analysis. As ACV is a highly polar substance, sample cleaning by conventional solvent extraction is not possible [1]. Solid-phase extraction on Sep-Pak Light C_{18} cartridge has been used in our laboratory for different polar substances, e.g. morphine glucuronides [13], benzoylergonine [14] and adenosine [15]. Application of serum spiked with ACV on a Sep-Pak Light C_{18} cartridge gave incomplete absorption. If up to 1 ml of plasma was mixed with the same volume of saturated sodium chloride, the absorption was complete for both ACV and CMMG. Plasma proteins were removed by washing with 0.5 ml of a 50% saturated sodium chloride solution. No ACV or CMMG was lost in this step. ACV and CMMG were eluted with 250+750 μl of a 3% acetonitrile solution in 38 mM phosphoric acid. The first 250 μl of the eluate did not contain any ACV or CMMG and was

discarded. More than 90% of both ACV and CMMG were recovered in the fraction from 250–1000 μl. At least 100 μl of the eluate could be injected onto the HPLC column without any problem, thus making it possible to further increase the sensitivity, if necessary. The eluates could be stored at room temperature for at least 24 h without any change in concentration. Guanosine, a nucleoside of which acyclovir is an analogue, has been injected on the HPLC system and found to have a retention time of 1.35 min (ACV: 1.70 min; CMMG: 1.50 min).

This HPLC method has been used since 1991, mostly for the analysis of serum samples from kidney and liver transplant recipients receiving oral ACV as a prophylactic agent against CMV infections. The predose ACV concentrations have ranged from 1 to 20 μ*M*. A representative example of the day-to-day variation during regular therapy for several months is shown in Fig. 4. So far, we have had no problems with interfering peaks in the analysis of more than 400 serum samples collected from organ transplant recipients who receive several other drugs, such as cyclosporine, azathioprin, prednisolone, sulfametoxazol, trimetoprim, nifedipin and furosemid.

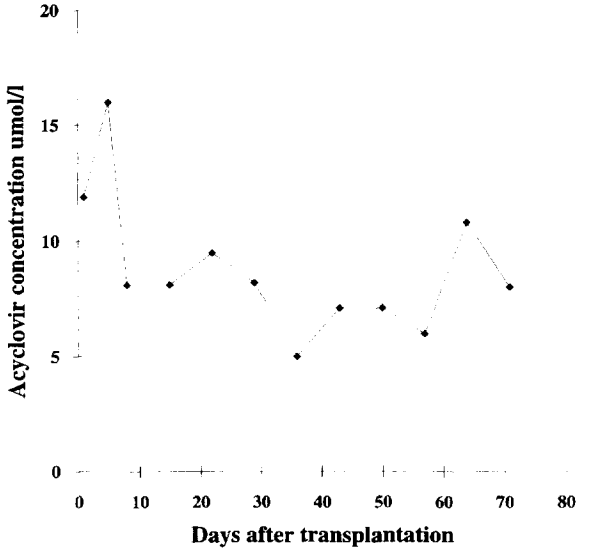

Fig. 4. Trough concentrations of ACV in serum in a renal transplant recipient receiving a daily 800-mg dose of ACV.

The relative proportions of ACV and CMMG varied considerably among the patients. The highest levels were found in patients with renal dysfunction. The possible relationship between neurotoxic symptoms and the amount of CMMG is currently under investigation.

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