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Analysis of the antiviral drugs acyclovir and valacyclovir-hydrochloride in tsetse flies (*Glossina pallidipes*) using LC–MSMS

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ABSTRACT

A new simple, sensitive and precise liquid chromatography–tandem mass spectrometry method has been developed and validated for the determination of valacyclovir-HCl and acyclovir in tsetse flies (*Glossina pallipides*). Tsetse flies were extracted by ultrasonication with acidified methanol/acetonitrile, centrifuged and cleaned up by solid phase dispersion using $MgSO_4$ and MSPD C_{18} material. Samples were analysed using a Waters Alliance 2695 series HPLC with a C_{18} Gemini analytical column (150 mm × 4.6 mm × 5 μm) and a guard cartridge column connected to a Waters Quattro-Micro triple-quadrupole mass spectrometer. The isocratic mobile phase consisted of methanol:acetonitrile:water (60:30:10, v/v/v) plus formic acid (0.1%) at a flow rate of 0.25 ml/min. The precursor > product ion transition for valacyclovir (m/z 325.1 > 152) and acyclovir (m/z 226.1 > 151.9) were monitored in positive electrospray multiple reaction monitoring mode. The method was validated at fortification levels of 0.5, 1 and 2 μg/g. The range of calibration for both drugs was 0.45–4.5 μg/g. The overall accuracy of the method was 92% for valacyclovir and 95% for acyclovir with corresponding within-laboratory reproducibilities of 4.4 and 3.4%, respectively. Mean recoveries were above 80% for both drugs and repeatability ranged from 0.7 to 6.1%. For both drugs the limits of detection and quantification were 0.0625 and 0.2 μg/g, respectively. The method was applied in experiments on the mass rearing of tsetse flies for sterile insect technique (SIT) applications, in which the flies were fed with blood meals containing acyclovir or valcyclovir-HCl prior to analysis to assess effects on *Glossina pallidipes* Salivary Gland Hypertrophy syndrome.

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1. Introduction

Tsetse flies (Diptera: *Glossinidae*) are vectors for trypanosomes, the etiological agents for two major diseases, human African trypanosomosis (sleeping sickness) and animal African trypanosomosis (nagana). Through systematic and thorough prevention and control activities, sleeping sickness was on the brink of elimination in the 1960s. However, due to deteriorating control strategies, there was a subsequent increase in the disease, with human infection rates estimated at 300,000–500,000 people with up to 60 million others at risk in 36 African countries [1]. The disease has been ranked 7th in sub-Saharan Africa in terms of disability-adjusted life years, and is the second globally ranked parasitic disease after malaria [1]. Renewed control efforts decreased the number of new cases by the year 2004 to 50,000–70,000 cases [2]. Besides human suffering, trypanosomosis causes an estimated economic loss of US$ 4.5 billion per annum. Thus, tsetse flies and the trypanosome infections they transmit are one of the greatest reasons for poverty and perpetual underdevelopment in sub-Saharan Africa. Fortunately, tools exist to combat the disease through vector control [1] such as the sterile insect technique (SIT), an important component of an integrated pest management strategy [3]. SIT involves mass production and release of sterile male tsetse flies. An example of the effectiveness of the technique is the elimination of *Glossina* in Zanzibar [4].

However, a syndrome known as *Glossina pallidipes* Salivary Gland Hypertrophy is an obstacle to SIT. This syndrome is associated with reduced fecundity and, subsequently, sterility in infected flies [5]. If not addressed, the syndrome presents a major impediment to the mass rearing of tsetse flies, which is a prerequisite for SIT, and therefore detrimentally affects tsetse control as a measure for eradication or control of sleeping sickness, with consequent immense health and economic implications.

One possibility for the mitigation of this problem is to use the antiviral drugs acyclovir (9-[2-hydroxyethoxy]-methylguanine)

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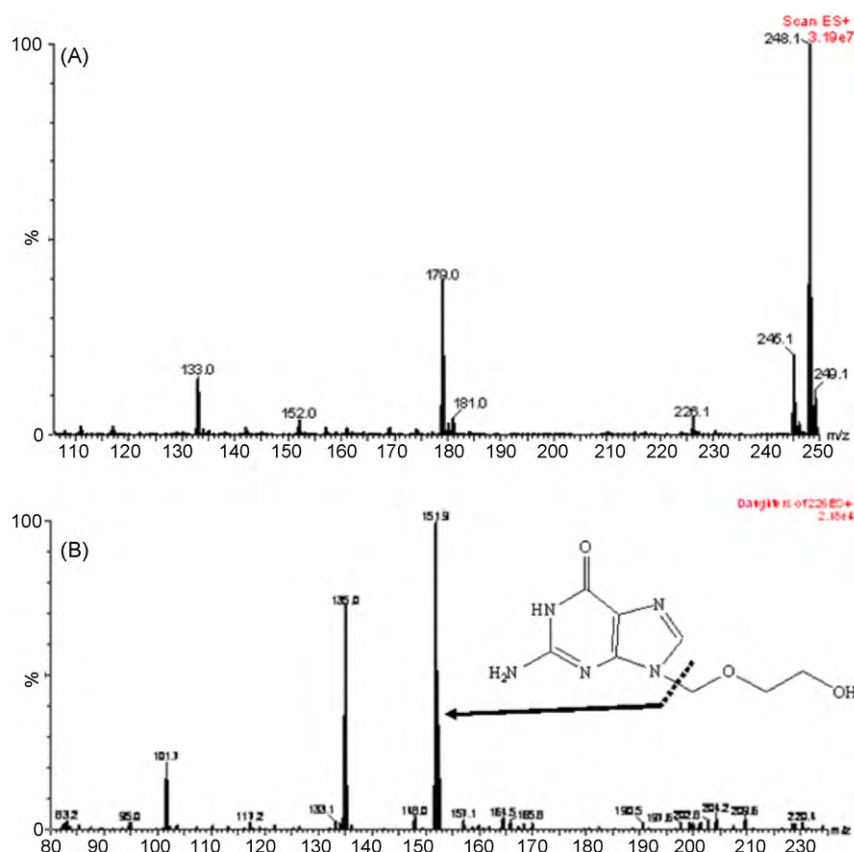

Fig. 1. Mass spectra of acyclovir showing: (A) the precursor ion at *m*/*z* 226.1 and (B) product ions at *m*/*z* 151.9 and 135 used as the quantification and qualifier ions, respectively.

(Fig. 1) or its L-valyl ester pro-drug valacyclovir (L-valine-2-[(2-amino-1,6-dihydro-6-oxo-9H-prin-9yl) methoxy] ethyl ester) (Fig. 2). These drugs are commonly used to treat herpes viral infections [6,7]. The *Glossina pallidipes* Salivary Gland Hypertrophy virus and herpes virus exhibit some genetic resemblance [8]. The pharmacokinetics/bioavailability of these drugs in vertebrates is well documented. For example valacyclovir, a known pro-drug of acyclovir, is almost completely converted to acyclovir when administered orally [6,7,9,10]. However, the pharmacokinetics of these drugs in invertebrates such as tsetse flies is not known.

Information on the behaviour and efficacy of these drugs for the control of the *Glossina pallidipes* Salivary Gland Hypertrophy syndrome could be obtained by orally feeding tsetse flies with a blood meal containing acyclovir or valacyclovir, followed by analytical chemistry to assess the drug profiles. This is a novel area of study that would support the SIT component of integrated pest management. Patil et al. [11] validated a reversed-phase liquid chromatographic method with ultra-violet/visible detection (LC–UV–vis) to determine valacyclovir and its degradation products including acyclovir in bulk drugs. Bras et al. [7] used high performance liquid chromatography with diode array detection (HPLC-DAD) to study the comparative bioavailability of acyclovir following treatment of herpes simplex infected human patients using oral acyclovir and valacyclovir. Granero and Amidon [9] also used HPLC-UV to study the oral bioavailability of valacyclovir in humans. Recently, Yadav et al. [12] studied the stability of valacycovir and acyclovir in human plasma using liquid chromatography–tandem mass spectrometry (LC–MSMS). All these studies involved vertebrates.

The objectives of this study were to develop an analytical method for the two antivirals, acyclovir and valacyclovir-HCl, in tsetse flies by LC–MSMS, using acyclovir and valacyclovir-HCl technical material as analytical standards, including a suitable sample preparation regime to facilitate analysis of the two drugs in tsetse flies, and to apply the method to analyze tsetse fly samples for the presence of acyclovir and valacyclovir-HCl in tsetse flies that were previously fed blood containing the drugs. LC–MSMS was chosen as the detection method because of the sensitivity and specificity/selectivity of the technique.

2. Experimental

2.1. Chemicals and materials

Acyclovir (95% purity) and valacyclovir-HCl (96% purity), both purchased from Molekula (UK), were used to prepare standard solutions. Methanol, acetonitrile, acetic acid and formic acid were all HPLC grade and dimethyl sulfoxide (DMSO) was analytical grade. Nanopurified/Milli Q water was used throughout. Tsetse flies (fed either with blood meal containing one or other of the two compounds of interest or blank blood meal) were provided by the Entomology Unit of the IAEA laboratories in Seibersdorf, Austria. Mixed $MgSO_4$ and MSPD C_{18} material (Biotage, Uppsala, Sweden) were used for sample clean-up.

2.2. Preparation of standards

Stock standards of acyclovir (1.08 mg/ml) and valacyclovir-HCl (1.03 mg/ml) were prepared by dissolving 10.78 and 10.34 mg of the drugs, respectively in 5 ml DMSO with ultrasonication (5 min) then adjusting the volume to 10 ml with DMSO and mixing thoroughly by inversion. A 10 μg/ml intermediate standard mixture of the two drugs was prepared by measuring 92.5 and 97 μl of acyclovir and valacyclovir-HCl, respectively into a 10 ml volumetric flask and adding methanol to make up to the calibrated mark. The solution was then thoroughly mixed. Working standards were

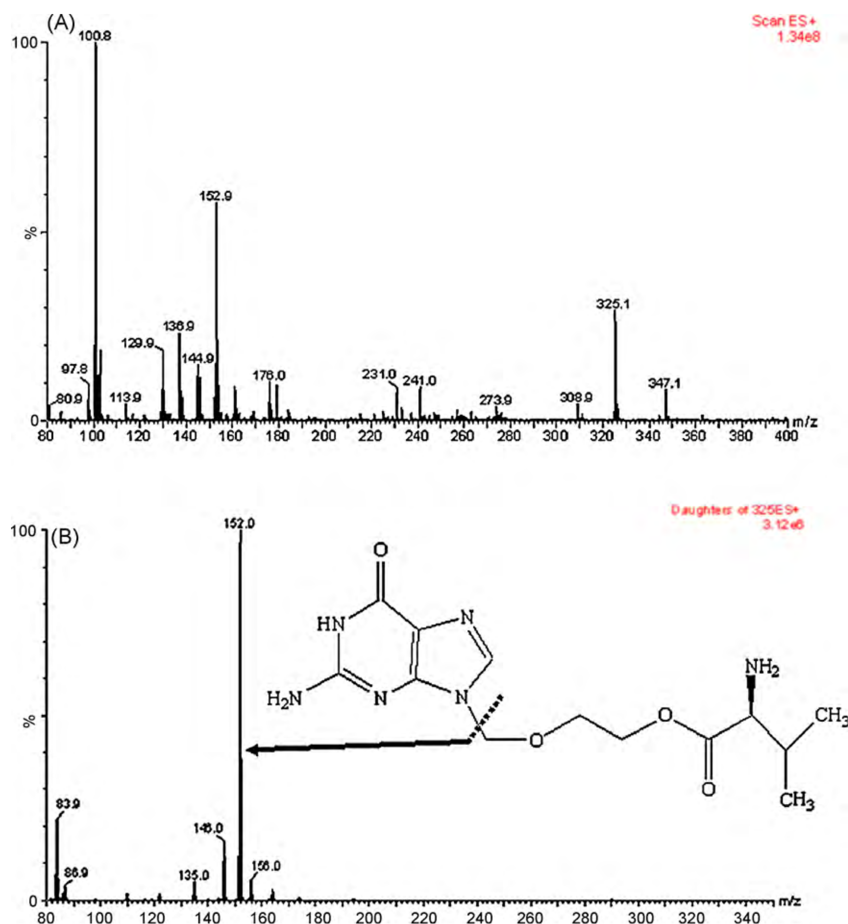

Fig. 2. Mass spectra of valacyclovir showing: (A) the precursor ion at m/z 325 and (B) product ions at m/z 152 and 83.9 used as the quantification and qualifier ions, respectively.

prepared by dilution in methanol from the intermediate mixed standard solution.

2.3. Sample preparation

Tsetse fly samples (~220 mg; 5–6 flies) were weighed into 25 ml glass conical test tubes and ground using a glass rod until a homogeneous paste was formed. The samples used for recovery studies were fortified at this point. At least two blank controls (non-fortified samples) were extracted for each recovery experiment to prepare matrix-matched standards. The samples were left to stand for 15 min before adding 5 ml of the extraction solvent (methanol:acetonitrile; 70:30, v/v containing 0.1% acetic acid). Capped test tubes containing the homogenized samples were then placed in 500 ml beakers containing water (approximately 1/3 full). The beakers were then placed in an ultrasonicator (VWR™, Model USC 300T) for 25 min.

After the ultrasonic extraction process, the extract was mixed on a vortex type mixer for 1 min. Where necessary, the solid material was disturbed/broken with a pasteur pipette. The contents were then transferred into 15 ml Sarstedt tubes and centrifuged (2600 × g, 4 °C, 5 min). The supernatant was transferred into new 15 ml Sarstedt tubes containing 1 g of a mixture of $MgSO_4$ and MSPD C_{18} (1.5:0.5, w/w). This mixture was vortex-mixed for 1 min and centrifuged (2600 × g, 4 °C, 10 min). The supernatant was filtered through Schleicher and Schuell filter papers (70 mm diameter) into 25 ml conical glass test tubes containing either 0.5 or 0.4 ml of DMSO. Recovery extracts and those prepared from samples of flies treated with the antivirals were filtered into the tubes containing 0.5 ml of DMSO, while the blank extracts that were used to prepare matrix-matched standards were filtered into the tubes containing 0.4 ml of DMSO. Before and after filtration, the filter papers were washed with 0.5 and 1 ml of the extraction solvent, respectively, in order to wet the filter papers and then rinse off any residue remaining. The extraction solvent was then evaporated from the filtrate using a TurboVap® LV evaporator (Zymark, Runcorn, UK) set at 50 °C under a stream of nitrogen, leaving the filtrate in DMSO. The blank extracts in 0.4 ml of DMSO were then spiked with 0.1 ml of appropriate dilutions of the antiviral mixed intermediate standard in order to obtain matrix-matched standards ranging from 0.45 to 4.5 μg/g in matrix. All samples were then filtered through 0.45 μm membrane filters prior to analysis by LC–MSMS.

2.4. LC–MSMS analysis

For sample analysis, the chromatography was achieved using a Waters Alliance HPLC 2695 series (Waters, UK) with separation on a reversed-phase C_{18} Gemini analytical column (150 mm × 4.6 mm × 5 μm, Phenomenex, USA, fitted with a guard cartridge). The mobile phase consisted of methanol:acetonitrile:water (60:30:10, v/v/v) plus formic acid (0.1%) at a flow rate of 0.25 ml/min and the column temperature was set at 45 (±5) °C. The run time was 8 min and the injection volume was 10 μl. The autosampler temperature was set at 22 (±2) °C. Detection and quantification was achieved using a Micromass Quattro-Micro™ triple-quadrupole mass spectrometer (Waters, UK) with electrospray ionization (ESI) interface in positive mode.

For initial set-up of the analytical method, the MSMS was equipped with a syringe pump loaded with a 500 ml Hamilton

Table 1
Quattro-Micro tune parameters used to determine suitable MSMS conditions for analysis of acyclovir and valacyclovir-HCl.

Parameter	Positive ESI
Capillary voltage	3.29 kV
Extractor	5 V
RF lens	0.1 V
LM 1 resolution	15.0
HM 1 resolution	15.0
Ion energy 1	0.5
Entrance	−1
Exit	2
LM 2 resolution	13.5
HM 2 resolution	13.5
Ion energy 2	0.5
Source temperature (°C)	150
Multiplier (V)	650
Desolvation temperature (°C)	400
Desolvation gas flow	650 l/h
Cone gas flow	50
Cone gas	Argon, p = 3.17e−3 bar
Syringe pump flow	30 μl/min

syringe to facilitate infusion experiments for characterizing and optimizing the ionization of the analytes and setting the parameters for data acquisition. The syringe pump was operated at a flow rate of 30 μl/min. The optimized mass spectrometric parameters are listed in Table 1. Multiple reaction monitoring (MRM) was employed with inter-scan and inter-channel delays set at 0.1 and 0.02 s, respectively. For acyclovir, the first quadrupole was set to transmit the pseudo-molecular $[M+H]^+$ ion at m/z 226.1 and the second quadrupole to transmit the product ions at m/z 151.9 and m/z 135. For valacyclovir, the pseudo-molecular ion at m/z 325.1 and the product ions at 152 and 83.9 were used. Nitrogen was used as the nebulizer/desolvation gas and argon as the collision gas. The LC–MSMS system was controlled by Masslynx software, and the results were processed by TargetLynx software (both from Waters, USA) and Microsoft® Excel (Microsoft Corporation, USA).

2.5. Method validation

The validation parameters examined were linearity, accuracy, recovery, precision (repeatability and reproducibility), sensitivity and specificity. For recovery and precision, blank samples were spiked at 3 concentration levels, with 37, 74, and 148 μl of a 3 μg/ml mixed standard solution (acyclovir and valacyclovir-HCl) to obtain a final concentration of 0.5, 1 and 2 μg/g, respectively. Five replicates were prepared for each fortification level. At least two blanks were prepared at each level. This was done by 2 independent operators, operator one on days 1 and 2 and operator two on day 3, to determine both repeatability and within laboratory reproducibility. Precision values below 20% were targeted. The linearity of the LC–MSMS response to acyclovir and valacyclovir was determined using matrix-matched standards at the concentrations 0.45, 0.9, 1.36, 1.8, 2.27, 2.72, 3.6, 4.5 μg/g. These were prepared by adding 100 μl of 1, 2, 3, 4, 5, 6, 8 and 10 μg/ml (in methanol) of mixed acyclovir and valacyclovir-HCl to 400 μl of negative (drug-free) tsetse fly matrix extract, prepared as described in Section 2.3. The matrix matched calibrators were similarly prepared but the calibration curve consisted of 7 points at the concentrations 0.45, 0.9, 1.36, 1.8, 2.27, 3.6, 4.5 μg/g.

To characterize the specificity and selectivity of the method, the possibility of interfering compounds was investigated by analyzing matrix-matched samples prepared using a blank sample (known to contain neither acyclovir nor valacyclovir) to ensure that there was no interference at the respective analyte retention times (Fig. 1). The specificity was further tested in fortified and incurred samples by acquiring two diagnostic daughter ions for each of the analytes, and by comparison of the ratios between the daughter ions, with a tolerance level set at ± 10% of the ratio in a calibration standard. The limit of detection (LOD) and limit of quantification (LOQ) were estimated by injecting decreasing concentrations of matrix-matched standards and measuring the response at a signal-to-noise ratio (S/N) of ≥3 and ≥10 for the LOD and LOQ, respectively. Analytes were typically identified on the basis of their characteristic diagnostic ion and their retention time, with additional confirmatory evidence provided by ion ratios (precursor, quantification and target ions).

Matrix effects (ionization suppression or enhancement) were investigated by comparing standards prepared in solvent with standards prepared in drug-free matrix extract.

2.6. Application of the method

Fitness for purpose was ascertained by analyzing 3 groups of tsetse flies, two of which were fed with a blood meal containing

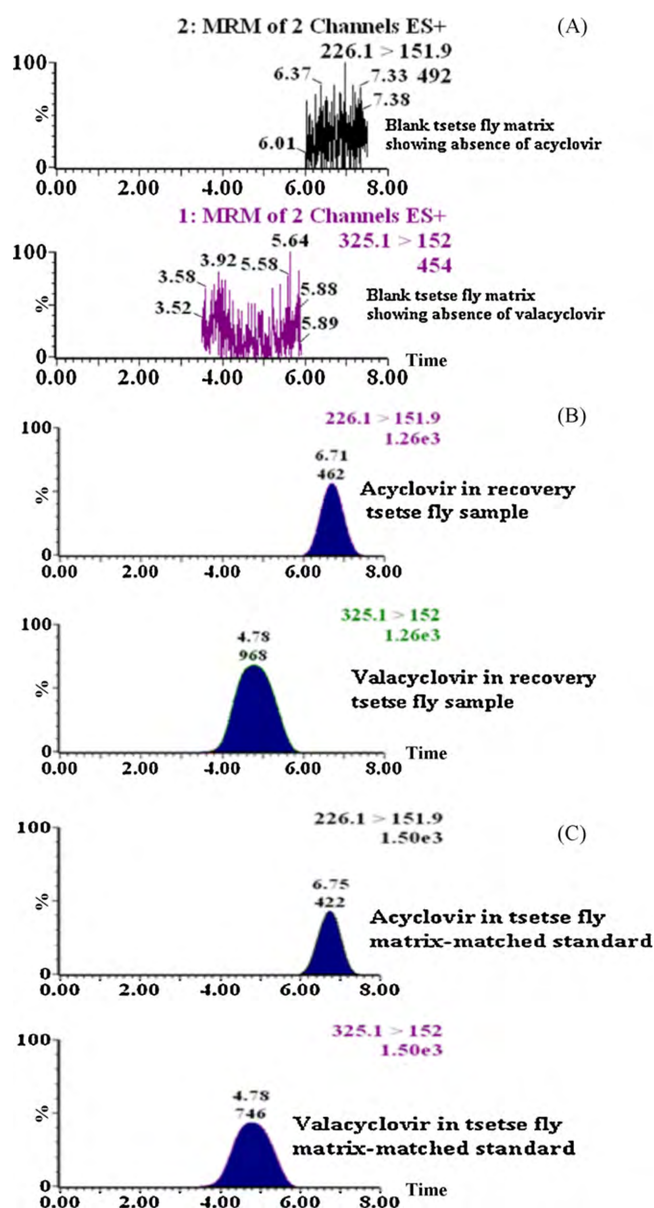

Fig. 3. MRM chromatograms showing (A) blank tsetse fly extract, (B) recovery of acyclovir and valacyclovir from blank tsetse flies spiked with 2 μg/g of the two drugs, and (C) acyclovir and valacyclovir in tsetse fly matrix at a concentration of 0.45 μg/g. Abundance figures are at top right of each chromatogram.

Table 2
Optimum LC–MSMS parameters for analysis of acyclovir and valacyclovir-HCl.

Drug	Parent ion (m/z)	Daughter ion (m/z)	Cone energy (V)	Collision energy (eV)	Dwell time (s)	Electrospray ionization mode
Acyclovir	226.1>	151.90[a]	32	12	0.15	Positive
		135.00[b]	32	26	0.15	Positive
Valacyclovir-HCl	325.1>	152.00[a]	40	15	0.15	Positive
		83.9[b]	40	20	0.15	Positive

[a] Quantification ion.
[b] Qualifier ion.

either acyclovir or valacyclovir while the third (control) group was fed with a blood meal that did not contain either drug. The latter group of flies was used to provide blanks/negative controls and for preparation of matrix-matched standards. Each group consisted of 75 flies. The drug was administered to the two experimental groups at a concentration of 300 μg/ml of the blood meal. The flies were fed three times a week and each fly consumed approximately 30 μl of the blood. Of the 75 flies, 60 were randomly selected for analysis. These were further sub-sampled to obtain the desired weights (∼220 mg). All flies were placed into 50 ml Sarstedt tubes and kept in a freezer at −20 °C before extraction and clean-up. Six replicate samples were prepared for each of the two groups of antiviral fed flies.

The maximum possible final concentration of each drug, assuming total absorption of the drugs and no metabolism before analysis, in the ∼5 tsetse fly samples required to make the analytical sample size (220 mg) was estimated at ∼45 μg/220 mg or ∼200 μg/g. It was anticipated that the actual measured values would be somewhat below this theoretical maximum. Extracted samples were, therefore, diluted (initially) 1:50 with blank matrix extract to bring the analyte concentrations into the calibration range.

3. Results and discussion

3.1. Sample extraction and clean-up

The extraction technique was optimized in terms of simplicity and speed. The simple solid phase dispersion clean-up using $MgSO_4$ and MSPD C_{18} material was adequate for this potentially difficult matrix, as can be seen in the chromatograms in Fig. 3. Initial problems with loss of recovery at the evaporation stage were overcome by using DMSO as a “keeper” rather than allowing the extract to evaporate to dryness.

3.2. Fragmentation of acyclovir and valacyclovir

Positive electrospray mode provided a better response than negative mode for both analytes. The pseudo-molecular ions at *m*/*z* 226.1 and 325.1 were determined as the protonated precursor ions $[M+H]^+$ for acyclovir and valacyclovir-HCl, respectively. Sodium adducts $[M+Na]^+$ were also seen at *m*/*z* 347 and 248 for valacyclovir and acyclovir, respectively. The corresponding daughter ions for the $[M+H]^+$ precursor ions were also determined (Table 2; Figs. 1 and 2). The most abundant ion in both cases was the ion at *m*/*z* 152 (or 151.9), which possibly represents a protonated guanine species also reported elsewhere [12]. These ions possibly result from neutral loss of 2-amino-3-methyl butanoic ethylene ester (143 Da) and a formaldehyde (30 Da) following γ-hydrogen rearrangement in the case of valacyclovir [13] and due to loss of a 2-methoxyethanol radical (74 Da) for acyclovir as proposed elsewhere [12]. We propose that the ions at *m*/*z* 135 in both compounds could result from loss of the NH_3 (or OH) radical from the protonated guanine (*m*/*z* 152 or 151.9). The other ions in the spectra are in agreement with a fragmentation pattern proposed for nucleosides elsewhere [14].

Given that acyclovir and valacyclovir are analogues and had closely related MSMS fragmentation patterns, chromatographic separation was essential. This was achieved using the Gemini C_{18} column (150 mm × 4.6 mm × 5 μm) with an isocratic mobile phase consisting of methanol:acetonitrile:water and formic acid. The flow rate of 0.25 ml/min was preferred to higher flow rates because it produced better signal response on the ESI-MSMS. The run time of 8 min facilitated rapid sample analysis and the retention times for valacyclovir and acyclovir were approximately 4.7 and 6.7 min, respectively.

3.3. Detection/quantification limits and linearity

The LOD ($S/N \geq 3$) and LOQ ($S/N \geq 10$) for both compounds were estimated at 0.0625 and 0.2 μg/g, respectively. Accuracy at the LOQ was 95% while the precision was 0.4%. The linearity of the response (peak area) to analyte concentration relationship was checked for both acyclovir and valacyclovir-HCl over a calibration range of 0.45–4.5 μg/g of the drugs in matrix. A correlation coefficient of linear regression (r^2) > 0.98 is normally regarded appropriate for analytical standards. The equations and correlation values for valacyclovir-HCl and acyclovir were $y = 1277x - 59.921$; $r^2 = 0.9924$ and $y = 1058.7x - 0.9257$;

Table 3
Recovery and precision of the method for tsetse fly matrix. The experiment was done on 3 different occasions, on the third occasion by a different operator, with 5 replicate fortified samples at 3 concentration levels.

Fortification level	Day 1 (op1)		Day 2 (op1)		Day 3 (op2)	
	Acy	Val	Acy	Val	Acy	Val
0.5 μg/g (n = 5)						
R1	102	91.3	95.5	96.3	95.9	82.3
R2	99.3	93.3	97.3	97.2	96.4	90.8
R3	99.2	90.5	96.2	98.6	96.7	89.3
R4	96.5	91.8	95.6	95.8	96.8	97.5
R5	98.7	89.8	95.3	96.7	95.5	91.6
Mean	99.0	91.0	96.0	97.0	95.4	90.2
Precision (CV), %	1.9	1.5	0.85	1.1	0.7	6.1
1 μg/g (n = 5)						
R1	97.6	93.2	96.1	95.0	97.2	87.9
R2	94.9	91.9	95.2	93.1	94.3	90.3
R3	90.8	94.4	95.2	94.4	92.9	86.6
R4	91.6	92.7	96.2	94.6	96.1	95.3
R5	98.9	91.1	93.7	93.9	98.7	85.4
Mean	95.0	93.0	95.0	94.0	95.8	89.1
Precision (CV), %	3.8	1.4	1.1	0.8	2.4	4.4
2 μg/g (n = 5)						
R1	101	99.8	99.4	93.6	80.9	76.1
R2	94.5	94.6	97.5	93.5	84.6	78.9
R3	96.0	95.0	97.2	95.2	84.0	83.0
R4	95.6	94.5	97.8	93.9	84.3	87.0
R5	96.9	98.3	96.3	93.2	81.7	86.0
Mean	97.0	96.0	98.0	94.0	81.8	82.2
Precision (CV), %	2.4	2.5	1.2	0.83	2	5.6
Summary	ACY	VAL				
Overall mean (days 1–3)	94.7	91.7				
Overall precision % (days 1–3)	3.4	4.4				

ACY: acyclovir; VAL: valacyclovir; R: replicate.

Table 4
Data showing levels of acyclovir (m/z, 226.1 > 151.9) and valacyclovir-HCl (m/z, 325.1 > 152) in matrix of tsetse flies that were fed with a blood meal containing acyclovir and valacyclovir-HCl (n = 6 per group of flies).

Sample replicate	Acyclovir-fed flies		Valacyclovir-fed flies	
	Acyclovir (μg/g)	Valacyclovir-HCl (μg/g)	Acyclovir (μg/g)	Valacyclovir-HCl (μg/g)
1	70.87	ND	41.3	ND
2	68.3	ND	35.98	ND
3	53.2	ND	46.8	ND
4	36.3	ND	45.4	ND
5	59.3	ND	45.2	ND
6	70.8	ND	41.3	ND
Mean (%)	59.8	N/A	42.7	N/A
SD	13.5	N/A	3.98	N/A

ND: none detected; N/A: not applicable.

r^2 = 0.983, respectively. Good linearity was obtained for both compounds.

3.4. Selectivity, specificity and matrix effects

Specificity was demonstrated by identifying the analytes based on the precursor and product ions as well as the relative retention times (compared to the standards). Ion ratios in matrix-matched calibrators and sample extracts typically matched each other to within 10%. For selectivity, following injection of individual analytes in matrix, peaks with acceptable S/N ratios were obtained at the retention times of both analytes, with no interfering peaks obtained at the respective retention times the in blank tsetse fly extract, as shown in Fig. 3.

To investigate possible matrix effects, including ionization, suppression or enhancement, the signal responses from standards prepared in solvent and those prepared in matrix extract were compared. The response was generally higher in “solvent standards”. However, the sensitivity of the method using matrix-matched standards was acceptable with a good signal response observed at a concentration of 0.0625 μg/g. In the final assay protocol, matrix/ionization effects were minimized by first optimizing the chromatographic and MS parameters to provide a strong signal for the analytes in extracted matrix, and by quantification using calibrators prepared in extracted negative matrix by the same method as the samples, thus compensating for matrix effects.

Initially we encountered problems (poor response) when the ESI negative mode was used (although direct infusion of the standards in the same mode produced very good response). The ESI positive mode was found to provide a better signal for the analytes in matrix. Also, lower mobile phase flow rates (0.25 ml/min) gave us better response than higher ones (e.g. 0.5 ml/min). Furthermore, use of formic acid provided better responses than mobile phase with or without acetic acid. Results from formic acid and ammonium formate were similar, but ammonium formate was eliminated to avoid the unnecessary use of buffer salts (which in any case required formic acid for pH adjustment).

3.5. Recovery, accuracy and precision data

The recovery values obtained for both acyclovir and valacyclovir-HCl are summarized in Table 3. Three concentration levels, 0.5, 1 and 2 μg/g were used as the low, middle and high concentrations. Recoveries obtained in preliminary studies (data not presented) including higher fortifications at 5, 6 and 12 μg/g did not show observable differences from values obtained at 2 μg/g fortication level. Thus, we chose to omit the higher concentrations and focus on the concentration range in which we expected the analytical results of the diluted samples to lie. The highest concentration for recovery estimation (2 μg/g) was not, therefore, at the top of the linear/standard curve as recommended elsewhere [15]. Satisfactory recoveries were obtained for both compounds at all fortification levels. Of all the 45 replicates used, only two were below 80% recovery (76.1 and 78.9%) otherwise all recovery levels ranged from 81 to 102% (Table 3). Accuracy was within 15% of the true values for both drugs, especially on days 1 and 2, as recommended for a variety of bioanalytical samples [15]. Although accuracy values on day 3 deviated by 18% from the true value, the overall accuracy levels for all 3 days were close to 100% (94.7 and 91.7% for acyclovir and valacyclovir, respectively), with corresponding within-laboratory reproducibility values of 3.4 and 4.4%, respectively. Precision values for both drugs were well within

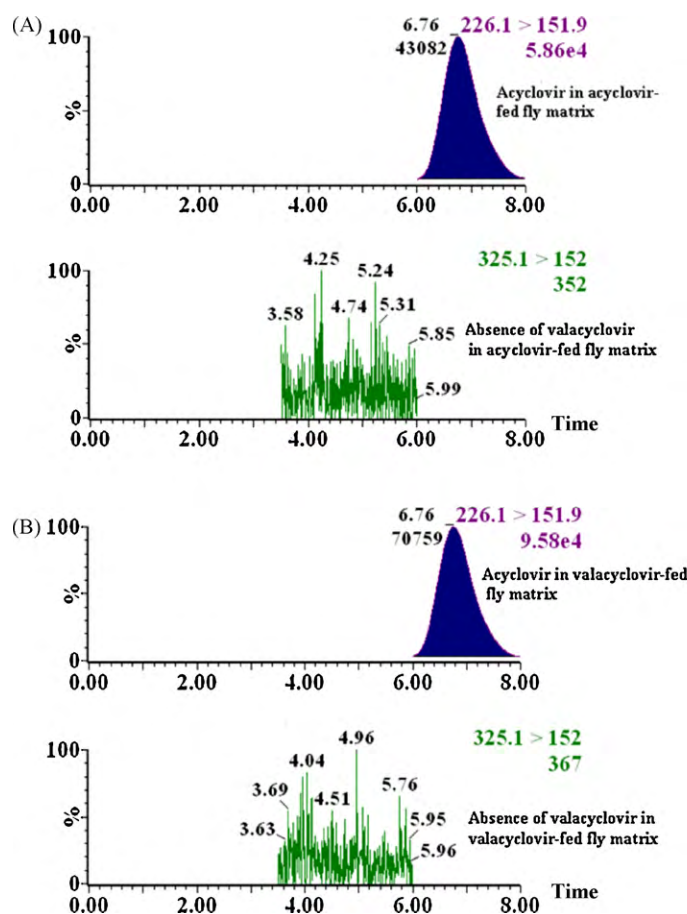

Fig. 4. MRM chromatograms showing presence/absence of acyclovir (m/z 226.1 > 151.9) and/or valacyclovir-HCl (m/z 325.1 > 152) levels in tsetse flies fed with blood meals containing (A) acyclovir, and (B) valacyclovir. The concentration of acyclovir in acyclovir-fed flies shown in this chromatogram was ~71 μg/g while the concentration of acyclovir in valacyclovir-fed flies was ~41 μg/g. Abundance figures are at top right of each chromatogram.

the 15% tolerance level recommended for bioanalytical samples [15].

The marginally poorer results for the validation experiment performed by operator 2 on day 3 may be explained by the fact that operator 2 was a temporary intern in our laboratory and was unfamiliar with the method and the instrumentation. However, we have included this data as an indicator of the robustness of the method under less than ideal circumstances.

The inclusion of suitable deuterated (or similar) internal standards would undoubtedly improve the performance of the method in terms of accuracy and precision by compensating for analyte losses during extraction and clean-up and also for variations in ionization efficiency in the mass spectrometer. However, the recoveries and precision (Table 3) obtained were satisfactory for the purpose of our research. Internal standards were considered, in this case, to be an unnecessary added expense.

3.6. Analysis of tsetse fly samples fed with a blood meal containing acyclovir and valacyclovir-HCl

To demonstrate the application of the method and its fitness for purpose, some results from part of an experimental protocol applied in the research project are presented in Table 4. The levels (mean ± SD, $n = 6$) of acyclovir detected in acyclovir-fed and valacyclovir-fed flies were 60 (±14) μg/g, and 43 (±4) μg/g, respectively (Table 4). No valacyclovir was detected in either set of flies at the method performance limits (Fig. 4). The presence of acyclovir in the tsetse flies that were fed with a blood meal containing valacyclovir and the absence of valacyclovir in both sets of flies are consistent with reports in the literature that valacyclovir is a pro-drug for acyclovir and that in vertebrates almost all of the valacyclovir is converted to acyclovir [6,9,10,16]. These results suggest that the same transformation occurs when the drugs are used in tsetse flies. The full results of the research to investigate the use of the antiviral drugs in mass tsetse rearing will be presented elsewhere.

4. Conclusion

A new simple, sensitive and precise LC–MSMS method has been developed and validated for the determination of valacyclovir-HCl and acyclovir in tsetse flies (*Glossina pallipides*). Prior to analysis, samples were prepared by extraction with a mixture of methanol, acetonitrile, water and acetic acid. Further clean-up of the samples was done using a mixture of $MgSO_4$ and MSPD C_{18} material. Both compounds could be detected at 0.0625 μg/g, while the quantification level was set at 0.2 μg/g. Acyclovir was detected in pooled tsetse flies that were previously fed with a blood meal containing either acyclovir or valacyclovir. Valacyclovir was not detected in either group. Besides presenting a new sample preparation protocol and analytical method, the findings suggest a rationale for the drug of choice needed to address the challenges presented by *Glossina pallidipes* Salivary Gland Hypertrophy syndrome to SIT. The method is being employed in further research to elaborate effective treatment regimes to support the mass rearing of tsetse for SIT.

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