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Journal of Chromatography B, 705 (1998) 79–85

JOURNAL OF CHROMATOGRAPHY B

Application of a standardized coextractive cleanup procedure to routine high-performance liquid chromatography assays of teicoplanin and ganciclovir in plasma

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Received 4 August 1997; received in revised form 18 September 1997; accepted 7 October 1997

Abstract

Deproteinization of plasma samples with acetonitrile followed by coextracting acetonitrile and lipophilic solutes with chloroform, as already proposed for methotrexate, is stressed as a general sample cleanup procedure for liquid chromatography of highly polar drugs, and was validated for two more applications: teicoplanin and ganciclovir. A dedicated ‘‘prevalidation’’ experimental design was used to assess performances of both assays, including sample preparation. Deviations from linearity were less than 10% over the ranges of 3.1 to 50 mg/l (teicoplanin) and 0.2 to 15 mg/l (ganciclovir), respectively, and limits of quantitation were 0.09 and 0.01 mg/l, respectively. Mean chromatographic measurement R.S.D.s were 4.6% and 1.9%, respectively, with an additional mean cleanup R.S.D. of 2% for both. Mean analyte losses ascribable to cleanup were 6% and 2.5%, respectively from water, and 18% and 12%, respectively from the plasma matrix. © 1998 Elsevier Science B.V.

Keywords: Teicoplanin; Ganciclovir

1. Introduction

The assay of drugs in plasma or serum by reversed-phase high-performance liquid chromatography (RP-HPLC) currently requires deproteinization. When chromatographing polar solutes, current deproteinization with a water-miscible organic solvent (most often acetonitrile), followed by injection of the supernatant mixture, is seldom usable because it results in sample less polar than mobile phase, and poor separation efficiency of most reversed-phase columns. To keep the aqueous composition of sample, perchloric or trichloroacetic acid can be used [1] at the expense of two dilution steps (addition of precipitating solution and desirable addition of a buffering solution before injection into the column) resulting in a loss of sensitivity.

We have previously described and validated a ‘‘coextractive cleanup’’ technique for a polar drug, methotrexate and its hydroxylated main metabolite [2]. It consisted of acetonitrile deproteinization, followed by extracting acetonitrile (together with lipophilic solutes) from the supernatant with chloroform, and RP-HPLC of the aqueous phase. The present paper is to validate the extension of this

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PII S0378-4347(97)00499-4

cleanup technique to two other polar drugs: teicoplanin and ganciclovir.

The antibiotic teicoplanin is a mixture of five glycopeptide components (A2-1 to 5 and A3-1) differing by a variable fatty-acid lipophilic moiety. Being eliminated mainly by the kidneys, it requires therapeutic drug monitoring (TDM) in renal failure [3]. It has been assayed by microbiological methods, fluorescence polarization immunoassay [4] and HPLC [5–8], the last being more currently used for TDM because of good specificity and sensitivity [3], as well as cost-effectiveness [9].

Ganciclovir is an acyclic nucleoside mainly used for chronic antiviral treatment of immunodeficient patients (drug-induced immunodepression, AIDS). Elimination of this polar drug is again mainly renal, thus monitoring plasma level is useful in renal failure [10–13]. Monitoring is also used in case of therapeutic failure to differentiate how much is due to individual pharmacokinetics and how much to viral resistance [14]. Ganciclovir has been assayed with radioimmunoassay [15], enzyme immunoassay [16] and RP-HPLC [1,17,18].

2. Experimental

2.1. Reagents

Acetonitrile was from Merck–Clevenot (Nogent-sur-Marne, France) and chloroform from Carlo Erba (Rueil-Malmaison, France).

Teicoplanin was used from a 10 g/l stock solution obtained by dissolving Targocid 400 mg lyophilisate (Marion Merrell, Levallois-Perret, France) in water, ganciclovir as a 7.5 g/l stock solution made by dissolving Cimevan 500 mg lyophilisate (Roche, Neuilly-sur-Seine, France) in water, and aciclovir (internal standard for chromatography) as a methanolic ca. 200 mg/l solution obtained by extracting 200 mg Zovirax tablets (Wellcome, Issy-les-Moulineaux, France). For each analyte, a high-level sample was prepared in water as well as in blank plasma, to be serially diluted according to the validation design: to 10.0 ml of water or of plasma, either 50 μl of teicoplanin stock solution or 20 μl of ganciclovir stock solution was added.

2.2. Chromatography

The chromatographic apparatus was composed of an SP8810 pump and an UV100 spectrophotometer from Thermo Separation Products (Les Ulis, France) a Model 655A-40 autosampler and a D2000 integrator from Merck (Darmstadt, Germany). A LiChrocart 250-4 RP8, 5 μm, column from Merck was used for teicoplanin, and a Supelcosil ABZ+ column from Supelco (St. Quentin Fallavier, France) for ganciclovir.

2.3. Sample preparation

In the ganciclovir application, an internal standard was first added to each 0.5 ml assay sample (10 μl, i.e., 0.02 volume of the 200 mg/l solution of aciclovir in methanol). Then the following cleanup steps were common to both drugs. Two volumes (1 ml) of acetonitrile were forcefully pipetted into the center of sample surface to minimize clotting of plasma proteins, then briefly stirred with a vortex mixer. After centrifugation (3 min, 2000 *g*, room temperature) the deproteinized supernatant was transferred to a glass tube and four volumes (2 ml) of chloroform were added. After vortex-stirring by brief strokes until stable volume partitioning (this needs ca. 10 strokes at maximal speed), the aqueous supernatant layer (currently named ''extract'') was transferred to a sampler vial. For the teicoplanin application, the extract was injected as such. For ganciclovir, the extract finally had to be flushed with nitrogen at 80°C for 3 min in order to remove organic solvents.

2.4. Chromatographic measurement

2.4.1. Teicoplanin

The isocratic mobile phase was an acetonitrile–ammonium acetate, 20 m*M*, pH 4.4 (26:74) buffer, flow-rate 1.3 ml/min, UV detection at 220 nm, room temperature. Thirty μl of the sample were injected. Peak height of the main component (A22) was used for calculations.

2.4.2. Ganciclovir

The isocratic mobile phase was acetonitrile–ammonium acetate buffer 10 m*M*, pH 5 (2:98), flow-

rate 1 ml/min, UV detection at 254 nm, injected volume 30 μl, room temperature. Relative peak height of ganciclovir to internal standard was used for calculations.

2.5. Validation design and calculations

Experiments conformed to our usual prevalidation design [19]. Water and plasma samples at five concentration levels in geometric progression were prepared by serial dilutions so as to bracket the therapeutic range: 50, 25, 12.5, 6.25 and 3.125 mg/l for teicoplanin and 15, 5, 1.67, 0.55 and 0.185 mg/l for ganciclovir. Each sample cleanup was duplicated, each measurement was programmed in duplicate, and the whole design was duplicated in two successive assay sessions.

Experimental results were subjected to ANOVA with the VALPLAN spreadsheet program as previously described [19]. Chromatographic measurement imprecision was calculated as the Napierian logarithmic mean square (standard deviation estimate) of measurement duplicates, read as arithmetical relative standard deviation (R.S.D.) [19] on line f of Tables 1 and 2. Measurement imprecision was also separately estimated at each concentration. The contribution s^2(xtr) of sample cleanup (''extraction'' component) to the error variance was calculated from between-measures s^2[M] and between-extracts s^2[X] mean squares of within-cell nested ANOVA, as follows:

$$s^2[\mathrm{X}] = s^2[\mathrm{M}] + 2s^2[\mathrm{xtr}], \textit{ or equivalently}$$

$$s^2[\mathrm{xtr}] = \{s^2[\mathrm{X}] - s^2[\mathrm{M}]\}/2$$

where factor 2 is the number of measurement replications from the same extract [20]. Cleanup contribution to imprecision also is readable as R.S.D. on line h of Tables 1 and 2.

Percent deviation of measurements (bias) from ''analytical linearity'' was calculated only for plasma samples at all five levels, as:

$$\{(\text{mean measurement} - \text{expected response})/\text{expected-response}\}*100$$

the expected response being the arithmetical regression line forced through zero (i.e., with zero intercept). For comparison, bias was also calculated from the standard, mean-squares fitted, arithmetical regression line.

Significance testing conformed to the random nature of the design session factor [21] and to the nested structure of within-cell ANOVA [20]: denominators for F tests and ''residual'' variance used for the t test of regression slope are indicated in Tables 1 and 2.

3. Results

3.1. Teicoplanin (Fig. 1, Table 1)

The limit of detection (LOD) defined as three times the chromatographic noise was 0.03 mg/l, and the limit of quantitation (LOQ) defined as 10 times the noise was 0.09 mg/l (2.7 ng in 30 μl injected). Mean extraction recovery was 94% from water and 81.5% from plasma, i.e., 87% from plasma relative to water. Chromatographic measurement imprecision regularly decreased from 4.8% at 3.125 mg/l, to 1.6% at 50 mg/l in water samples, and respectively, from 8.8% to 2.5% in plasma samples (overall ANOVA estimate: 4.6%). Sample cleanup added a significant ''extraction'' variance component to the measurement variance, estimated as an additional cleanup R.S.D. of 2.1%. The slope of the common logarithmic regression line was significantly higher than 1 [$(b-1)=0.026$, $t_{20}=2.078$, $P=0.05$], and there was no significant curvature (F bilat $1/4=5.84$, $0.10<P<0.20$). Mean percent deviation (bias) of plasma measures from analytical linearity (regression forced through zero) ranged from 1.2 to 6.5 per cent, irrespective of concentrations. Deviations from the least-squares fitted regression line reached 19% at the lowest level.

3.2. Ganciclovir (Fig. 2, Table 2)

LOD was 0.003 mg/l, and LOQ 0.01 mg/l (0.3 ng in 30 μl injected). Mean extraction recovery was 97.5% from water and 88% from plasma, i.e., 90% from plasma relative to water. The overall Napierian logarithmic S.D. (arithmetical R.S.D.) of measurement ranged from 0.4% to 3.4% in water samples and from 0.5 to 3.1% in plasma, irrespective of

Table 1
Analysis of variance of teicoplanin assay prevalidation design

Components	DF	Var[ln]	%R.S.D.	F tests (denominator)		Remarks
				Vs. an ia	Vs. (g)	
Factorial analysis						
Total	79	1.0328823				
a BTW sessions	1	0.00069555	2.64	–	0.23	
b′ BTW matrices	1	0.41274733	64.24	60.9 (k)	–	Serum/water = 86.6%
c BTW concentrations	4	20.2336839		–	–	($0.1<P<0.2$)
d BTW cells	19	4.28695267		–	–	
e Within cells	60	0.00242668	4.92	–	–	
f BTW measures	40	0.0021348	4.62	–	–	
g BTW extracts	20	0.00301043		–	1.4	($P>0.2$)
h Extraction component		0.00043781	2.09	–	–	
i iaCCN×MAT	4	0.01064205		5.4 (l)	–	($0.1<P<0.2$)
j iaCCN×SSN	4	0.01168971		–	3.92	$0.02<P<0.05$
k iaMAT×SSN	1	0.00677266		–	2.25	($P>0.2$)
l iatriple	4	0.00195567	–	0.65		
Analysis of regression						
m Linear regression	1	80.8635471		–	–	
n Curvature	1	0.06830107		5.8 (j)	–	($0.1<P<0.2$)
o Sigmoidicity	1	0.00051916		0.04 (j)	–	
p Nonparallelism	1	0.04226514		21.6 (l)	–	$P=0.02$
q Opposite curvature	1	0.0000219		0.011 (l)	–	
Common slope: $b=1.026$				2.08 (j[a])	–	$P=0.05$
Water samples: $b=1.049$						
Plasma samples: $b=1.002$						
Separate precision and bias estimates						
Concentrations (mg/l)	3.12	6.25	12.5	25	50	
R.S.D. (%):						
Water samples	4.80	4.50	3.39	3.85	1.67	
Serum samples	8.81	7.22	1.64	1.57	2.48	
Bias of plasma samples from arithmetical regression (%):						
Standard regression	19.3	3.57	−3.94	−2.44	0.72	
Without intercept	1.24	−3.49	−6.48	−3.0	1.20	

DF: Degrees of freedom, Var[ln]: variance estimation of Napierian logarithmic transforms, %R.S.D.: relative standard deviation, percent, BTW: between.
ia: Interaction, iaCCN×MAT×SSN: interaction of concentration, matrix and session factors.
[a] t test of $(b-1)$.

concentrations measured (overall ANOVA estimate: 1.9%). The additional R.S.D. of sample cleanup was 2%. The logarithmic slope was significantly lower than 1 [$(b-1)=-0.014$, $t_{20}=4.97$, $P<0.001$], the overall curvature of the logarithmic regression line again was not significant. Bias of plasma measures from ‘‘analytical linearity’’ regularly decreased from 11% to −0.04% as concentration increased, whereas deviations from the least-squares fitted regression line appeared both lower and random in this case.

4. Discussion

Both teicoplanin and ganciclovir have already been assayed by RP-HPLC after cleanup of acetoni-

Table 2
Analysis of variance of ganciclovir assay prevalidation design

Components	DF	Var[ln]	%R.S.D.	F tests (denominator)		Remarks
				Vs. an ia	Vs. (g)	
Factorial analysis						
Total	79	2.33191008				
a BTW session	1	0.00550181	7.4		4.8	(0.05<P<0.1)
b′ BTW matrices	1	0.23079142	4.8	12 000	–	serum/water=89.8%
c BTW concentrations	4	45.97828980		–		(highly significant)
d BTW cells	19	9.69387628		–		
e Within cells	60	0.00062079	2.5	–		
f BTW measures	40	0.00036351	1.9	–		
g BTW extracts	20	0.00113533		–	3.12	P=0.002
h Extraction component		0.00038591	1.96	–	–	
i iaCCN×MAT	4	0.00621450		7.39 (l)		(0.05<P<0.1)
j iaCCN×SSN	4	0.00148881		–	1.30	(P>0.5)
k iaMAT×SSN	1	0.00001900		–	0.017	
l ia triple	4	0.00084112		–	0.74	
Analysis of regression						
m Linear regression	1	183.910021	–	–		
n Curvature	1	0.00001583		0.01 (j)	–	
o Sigmoidicity	1	0.00006098		0.04 (j)	–	
p Nonparallelism	1	0.00187702		2.23 (l)	–	(0.2<P<0.5)
q Opposite curvature	1	0.01419839		16.88 (l)	–	P<0.001
Common slope: b=0.9759				4.97 (j[a])	–	P<0.001
Water samples: b=0.979						
Plasma samples: b=0.973						
Separate precision and bias estimates						
Concentrations (mg/l)	0.185	0.555	1.67	5.0	15	
R.S.D. (%):						
Water samples	0.58	0.68	0.39	3.39	1.00	
Plasma samples	1.725	2.63	3.13	1.75	0.47	
Bias of plasma samples from arithmetical regression (%):						
Standard regression	−2.82	4.0	−0.69	−0.18	0.02	
Without intercept	11.3	8.88	0.71	0.12	−0.04	

DF: Degrees of freedom, Var[ln]: variance estimation of Napierian logarithmic transforms, %R.S.D.: relative standard deviation, percent, ia: interaction, iaCCN×MAT×SSN: interaction of concentration, matrix and session factors. BTW: between.
[a] t test of $(b-1)$.

trile-deproteinized samples in the same way as the present one, teicoplanin with dichloromethane [3] and ganciclovir with diethyl ether [18]. Diethyl ether was not retained for two main reasons: (1) the underlying aqueous phase is more difficult to sample, and (2) extractive loss of polar analyte is high in such a polar solvent mixture, which accounts for the poor (34%) recovery [18]. Chloroform was preferred to dichloromethane because of its lower polarity and its higher density. Direct isocratic chromatography of the lower aqueous phase was found selective enough for teicoplanin, with even better sensitivity than in [3]. Concerning ganciclovir, which was eluted with a much more polar mobile phase, direct injection of the aqueous layer resulted in poor separation efficiency. It was found necessary to first heat it under nitrogen or vacuum in order to evaporate the small amount of organic solvents dissolved in water thus maximizing the polarity of the sample. However, this additional step can be a source of sample-to-sample

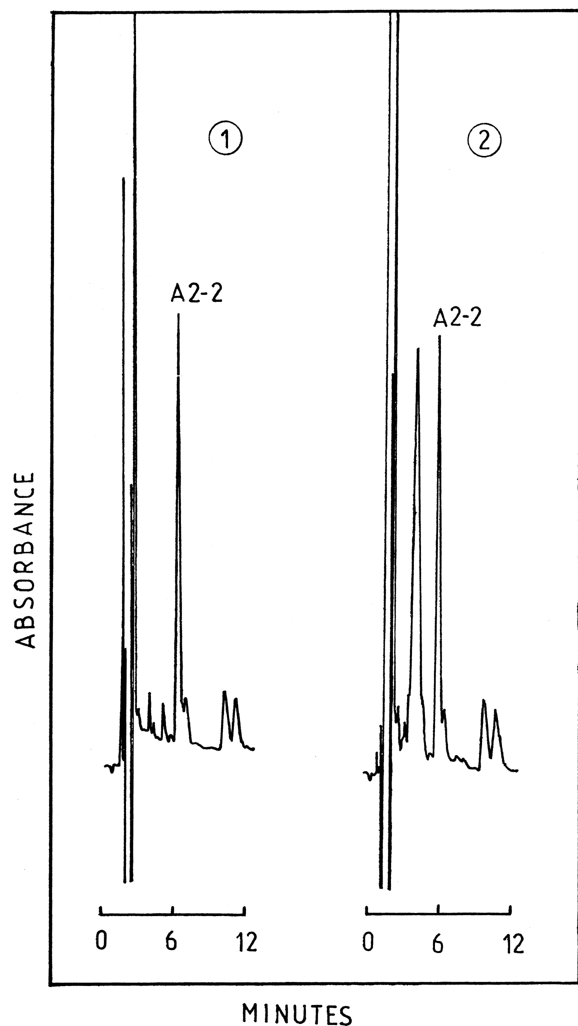

Fig. 1. Chromatograms of teicoplanin samples of median concentration (12.5 mg/l). (1) Water sample, (2) plasma sample. A2-2 is the main teicoplanin component, and the only one used for quantitation.

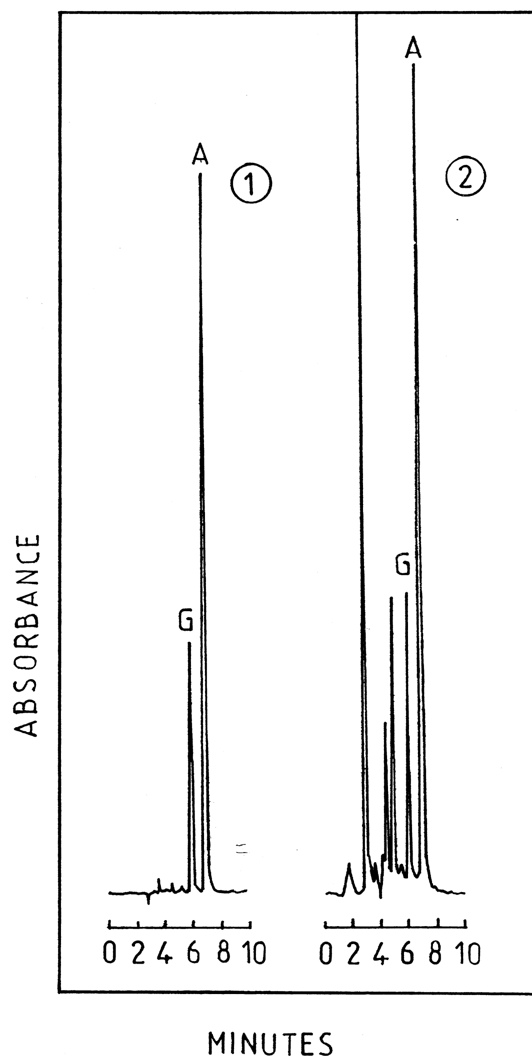

Fig. 2. Chromatograms of ganciclovir samples of median concentration (0.55 mg/l). (1) Water sample, (2) plasma sample. G: Ganciclovir; A: aciclovir used as internal standard.

variability due to uncontrolled sample concentration, which makes internal standardization mandatory. Loss of ganciclovir due to the present cleanup technique was low when applied to water samples (2.5%); an additional 6% loss from plasma may be best explained by analyte retention on the protein precipitate. Losses are somewhat higher for the more lipophilic teicoplanin: 6% from water and 13% more from plasma. The "extraction" component of between-extracts variance, expressed as R.S.D., was about 2% in both assays: the described coextractive cleanup procedure shows good repeatability for both analytes.

Measurements show better within-session repeatability for ganciclovir relative peak height at 254 nm (R.S.D. = 1.9%) than for teicoplanin absolute peak height at 220 nm (R.S.D. = 4.6%), but better between-session reproducibility for teicoplanin (R.S.D.s of 2.6% vs. 7.4%).

Direct proportionality between measures and con-

centrations (analytical linearity) is a common feature of spectrophotometric measurement due to the Beer–Lambert behavior of photometers. Since this allows the technical simplification of one-point calibration, it deserves validation. Regression analysis on logarithmic transforms was proposed in this purpose in the VALPLAN prevalidation design: analytical linearity is suggested when both logarithmic slope does not differ from 1 and there is no significant deviation from linearity (null hypothesis of regression tests). In both applications presented, the logarithmic slope differed significantly from 1, without significant curvature. However, for teicoplanin in plasma samples, slope was very close to 1 (1.002) and percent deviations from analytical linearity were lower than those from standard linear regression: lack of analytical linearity clearly has been caused mainly by water samples for unknown reasons. In the case of ganciclovir, lack of analytical linearity of plasma samples is confirmed by bias estimations, thus one-point calibrations may be suboptimal.

Previously published HPLC assays of teicoplanin showed comparable precision and similar [8] or lower [3,7] sensitivity. Fluorescent derivatization [7] did not enhance sensitivity over UV absorption. Taylor et al. [8] used solid-phase extraction, Awni et al. [3] a cleanup technique similar to ours. Published assays of ganciclovir were of lower [1] or similar [18] sensitivity. Boulieu et al. [1] used perchloric deproteinization without buffering the injected sample, a practice that we found prejudicial to RP columns. Hedaya and Sawchuk [18] used a cleanup method similar to ours, with diethyl ether instead of chloroform and larger solvent volumes, resulting in low (34%) recovery.

While chosen first for its operative simplicity proper to routine applications, the presented cleanup technique was also expected to be particularly reliable and robust: extraction of an observable amount of acetonitrile is a clear-cut endpoint, which had already shown reliable for an assay of methotrexate [2]. In the present work, extensive assessment of analytical performance with a dedicated experimental design shows that this fairly holds for other polar drugs.

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