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Determination of new anti-HIV agents, the *KR-V* series, in rat plasma using microbore high-performance liquid chromatography

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Abstract : We have developed a rapid, simple and precise high-performance liquid chromatographic (HPLC) method using an UV detection system for the determination of new anti-HIV candidates, nineteen *KR-V* compounds, in rat plasma. We used a analytical columnn of C_{18} (5 μm, 250×2.0 mm I.D.) and a mobile phase of water and ACN mixture (40/60, v/v). Under these conditions, all the *KR-V* compounds were readily separated from plasma with retention times of 4-12 min. The limits of quantitation for the 19 *KR-V* compounds were 15-30 ng/*ml*. The recoveries from the plasma were higher than 85% (C.V. <10%) with exception of *KR-V 2, 7* and *15*. The compounds *KR-V 2, 7* and *15*, containing ester moieties, were found to be unstable in plasma. This result suggests that esters, like *KR-V 2, 7* and *15*, should be excluded from future structure design studies of anti-HIV *KR-V* agents. In conclusion, the current HPLC method is a valuable analytical tool for investigating the pharmacokinetics of the *KR-V* series in rats.

Key words : HIV, HPLC, *KR-V* series, rat plasma.

Introduction

Acquired immunodeficiency syndrome (AIDS) is a clinically multifaceted disease induced following infection by the human immunodeficiency virus (HIV). Infection with HIV is usually followed by a period of clinical latency which, much later on, leads to the development of full-blown AIDS and death[1]. Since the discovery of AIDS in 1981 and the casual relationship to HIV in 1983, a number of different therapeutic strategies have been investigated[2].

Studies of the life cycle of the virus have identified several areas for therapeutic intervention, such as the class of reverse transcriptase (RT) inhibitors, the protease inhibitors, and their combination[3-5]. Most widely studied nucleoside or non-nucleoside RT inhibitors (NNRTIs) offered the first hope for increasing life expectancy in patients afflicted by the infection. However, emergence of viral resistance coupled with serious side-effects associated with the use of some nucleoside RT inhibitors (NRTIs) has resulted in only marginal success in combating disease progression[6].

Azidothymidine (AZT) and 1-[(2-Hydroxyethoxy)-methyl]-6-phenylthiothymine (HEPT) with a uracil nucleus were well known RT inhibitors. However, these compounds also have revealed several therapeutic disadvantages including severe toxicity and the occurrence of drug-resistant variants. In attempts to circumvent their cytotoxicity and the emergence of drug-resistant variants, the *KR-V* series of compounds have recently been synthesized by the Korea Research Institute of Chemical Technology, based on the uracil nucleus, like drugs such as AZT or HEPT (Fig 1). The present study was carried out to develop a simple and rapid high performance liquid

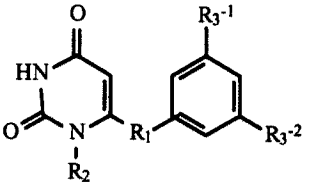

Fig 1. Basic structure of *KR-V* series.

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chromatography (HPLC) method for investigating pharmacokinetics of the *KR-V* series.

Materials and Methods

Chemicals

Nineteen *KR-V* compounds (purity > 99%) were investigated in this study. HPLC grade acetonitrile (ACN) and methanol (MeOH) were purchased from Burdick & Jackson Inc. (USA). All other chemicals were of reagent or analytical grade. Deionized water was used throughout the experiments.

Quantitative analytical method

1) HPLC system: A microbore HPLC (Hewlett Packard Co., USA) with a photodiode array UV detector (DAD) was used. The analytical column was a Capcell-pak C_{18} column (250 × 2.0 mm I.D., 5 μm, Shiseido, Japan); the flow rate was 0.2 ml/min and the column-oven temperature was 35°C. The injection volume was 5 μl. Optimal mobile phase conditions for the *KR-V* compounds were selected using an ACN-water gradient system and the UV wavelength was selected following a UV scan of the region 190-400 nm.

2) Extraction process: Deproteinization was examined using ACN or MeOH. An aliquat of serum was mixed with three volumes of ACN or MeOH. After vortex-mixing and centrifuging (2 min at 12,000 rpm), the supernatant was injected directly onto the column.

3) Stability: The stability of the *KR-V* series in a working solution (0.5 μg/ml MeOH) was studied for 0-, 1-, 2- and 3-day periods at room temperature and at 1°C. The stability of the compounds in rat plasma was also studied over 0, 1 and 2 weeks when stored at -20°C in a freezer.

Analytical parameters

1) Precision and accuracy: Precision and accuracy were assessed by performing replicate analyses (n=3) of samples spiked with 0.5 μg/ml for each of the *KR-V* series. Accuracy was evaluated as the percentage error [(mean of measured-mean of added concentration)/mean of added concentration] × 100, while the precision was given by the appropriate coefficients of variation[7,8].

2) Recovery: The extraction recovery was determined by comparing peak areas from drug-free plasma spiked with 0.5 μg/ml for each member of the *KR-V* series *versus* peak areas of the same concentrations prepared in MeOH injected directly onto the analytical column. Each sample was determined in triplicate.

3) Determination of the limits of quantitation (LOQ) and detection (LOD): The LOQ or LOD was determined from the signal-to-noise ratio (S/N ratio). The LOQ was defined as that concentration of the compounds resulting in an S/N ratio of 10 and LOD was defined as the sample concentration resulting in an S/N ratio of 4[9].

Results

Development of the quantitative analytical method

Optimal conditions of mobile phase and wavelength for *KR-V* compounds were obtained using C_{18} column under which all the compounds were eluted within 12 min (Table 1). MeOH, as an extracting and deproteinizing solvent, produced a better resolution for baseline noise and recovery than ACN (Table 2). In the blank plasma samples, no endogenous peaks interfered at the retention times of *KR-V* series. Although most of the *KR-V* compounds showed higher recoveries (>85%), *KR-V 2, 7* and *15* showed much lower recoveries 3.1, 20.2 and 36.9%,

Table 1. Optimal chromatographic conditions for the quantitative analysis of *KR-V* series

KR-V No.	Mobile phase		UV wavelength (nm)
	Fraction (ACN/Water, %)	Retention time (min)	
1	60/40	10.6	270
2	60/40	7.3	267
3	60/40	8.7	265
4	60/40	7.0	270
5	60/40	9.0	270
6	60/40	7.5	270
7	60/40	6.3	270
8	50/50	6.6	270
9	50/50	6.3	270
10	60/40	8.1	267
11	60/40	6.7	263
12	60/40	8.6	265
13	60/40	7.3	256
14	60/40	7.1	261
15	60/40	5.5	263
16	60/40	6.6	256
17	60/40	7.1	310
18	50/50	7.9	265
18-1	60/40	5.3	265

ACN, acetonitrile

Table 2. Extraction procedure recoveries for *KR-V* series in acetonitrile or methanol

KR-V No.	Recovery (%)	
	ACN	MeOH
1	96.4	99.5
2	–	3.1
3	100.5	102.8
4	99.1	104.1
5	110.1	115.0
6	108.7	110.6
7	–	20.2
8	84.2	89.6
9	113.0	115.0
10	117.7	118.1
11	113.7	115.5
12	101.6	104.2
13	107.1	105.1
14	118.1	105.4
15	–	36.9
16	135.4	118.5
17	106.2	101.3
18	112.3	110.2
18-1	109.6	105.7

respectively. When the well known esterase inhibitor, potassium fluoride, was added to the plasma, the recoveries of *KR-V 2, 7,* and *15* significantly increased up to 105, 97.4 and 96.0%, respectively.

Stability

All *KR-V* compounds were readily soluble in MeOH or ACN, but poorly soluble in water. All the *KR-V* series in MeOH or ACN solutions (working solution) were stable for at least 3 days at room temperature or under 1°C storage conditions (Table 3). The stability in plasma stored at -20°C in a freezer is shown in Table 4. The recoveries of the *KR-V* compounds, except for *KR-V 2, 7* and *15* having an ester linkage, were stable in rat plasma stored at -20°C, at least for 2 weeks.

Precision and accuracy

Precision and accuracy for the determination of the *KR-V* compounds (500 ng/ml spiked-serum samples) did not exceed 15% except *KR-V 2, 7* and *15* (Table 5). The coefficient of variation (C.V.) of *KR-V 2, 7* and *15* was 17.8, 23.1 and 3.9%, and the corresponding accuracy was 93.9, 72.0 and 32.1%, respectively.

Table 3. Stabilities of *KR-V* series in acetonitrile or methanol for 3 days

KR-V No.	Concentration (% for initial level)			
	ACN		MeOH	
	Room temperature	1°C	Room temperature	1°C
1	114	89	107	100
2	114	78	108	99
3	107	86	107	99
4	111	84	107	95
5	110	89	107	95
6	108	88	108	96
7	103	86	110	95
8	103	91	105	95
9	109	87	106	96
10	110	93	107	92
11	110	86	106	94
12	106	85	107	98
13	113	89	111	99
14	116	89	109	100
15	111	89	108	98
16	109	88	108	102
17	107	87	106	95
18	111	86	109	98
18-1	103	90	108	99

Table 4. Stabilities of *KR-V* series in plasma stored at -20°C for 2 weeks

KR-V No.	*KR-V* series concentration (% of initial level)	
	1 week	2 week
1	94.9	102.7
2	–	–
3	85.5	79.6
4	88.2	89.6
5	95.1	110.1
6	107.2	106.2
7	41.4	–
8	90.7	90.9
9	104.6	96.9
10	97.9	97.2
11	86.6	88.6
12	92.4	90.0
13	102.6	116.7
14	91.5	81.7
15	14.4	20.2
16	97.8	99.5
17	96.5	93.1
18	89.2	91.6
18-1	95.4	95.2

Table 5. Precision and accuracy of the HPLC method for *KR-V* series

KR-V No.	Observed concentration (ng/ml)[a]	Accuracy[b] (%)	CV (%)
1	490 ± 44.0	2.1	9.0
2	31 ± 5.5	93.9	17.8
3	538 ± 28.0	7.5	5.2
4	536 ± 25.9	7.2	4.8
5	457 ± 30.2	8.6	6.6
6	52 ± 17.5	9.5	3.9
7	14 ± 2.4	72.0	23.1
8	495 ± 6.3	1.0	0.7
9	426 ± 13.3	14.6	3.1
10	465 ± 45.4	6.9	9.8
11	528 ± 29.2	5.6	5.5
12	491 ± 27.7	1.7	5.6
13	486 ± 19.0	2.9	3.9
14	443 ± 13.3	11.5	3.0
15	339 ± 13.2	32.1	3.9
16	435 ± 24.6	12.9	5.7
17	429 ± 26.9	14.2	6.3
18	476 ± 28.2	4.8	5.9
18-1	487 ± 14.6	2.6	3.0

CV, coefficient of variance
[a]Mean ± S.D. (n=3)
[b]Percentage difference from the theoretical value (500 ng/ml).

Limit of quantitation and detection

The LOQ for the *KR-V* compounds ranged from 15 to 30 ng/ml and the LOQ ranged from 6 to 13 ng/ml.

Discussion

The purpose of present study was to develop a HPLC method for investigating the pharmacokinetics of the new series of synthetic anti-AIDS agents, *KR-V* series, in rats. A total of nineteen *KR-V* compounds were analyzed by a microbore reversed-phase system with UV detection using a C_{18} column and a mixture of water and ACN as the mobile phase. Under these conditions, all samples were rapidly eluted within 12 min. The LOQ for the compounds ranged 15-30 ng/ml. MeOH extraction was used because MeOH gave a more selective and higher recovery than ACN. Precision and accuracy were less than 15% for the *KR-V* series, except for *KR-V 2, 7* and *15* from plasma were very poor, the esterase inhibitor, potassium fluoride, increased the recoveries of all three compounds. The results suggests the possibility of enzymatic hydrolysis by plasma esterase(s) in rats. Due to their instability in plasma, the *KR-V* compounds with ester moieties should be excluded from the structure modifications associated with the design of new HIV *KR-V* agents. Therefore, the present analytical technique is considered to be sufficient for the determination of *KR-V* compounds in investigations of their pharmacokinetic properties.

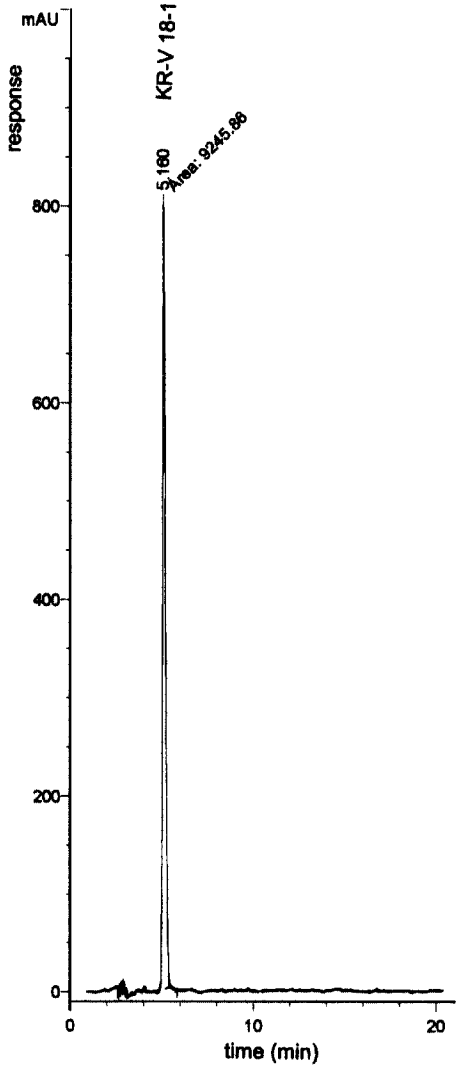

Fig 2. Chromatogram of a standard solution containing *KR-V* 18-1 (1 μg/injection). The mobile phase was a mixture of water and acetonitrile (40 : 60%). Detection wavelength was 265 nm.

Several methods for the determination of AZT in the plasma have been reported using HPLC systems with ion-pair chromatography[10,11], solid phase extraction[12] or

fluorescence detection[13]. In these reports, the LOQ values of AZT in the plasma were 5-50 ng/ml. Although the current microbore-HPLC method has a simple liquid extraction process, LOQ values for various types of *KR-V* compound were not inferior to the eralier methods for AZT.

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HPLC를 이용한 랫드혈장내 새로운 항HIV제 KR-V series의 분석법

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(2000년 9월 6일 게재승인)

국문초록 : 최근 새로 개발중인 항AIDS치료제 KR-V 시리즈를 대상으로 HPLC-UV 검출법을 이용해 랫드 혈장중에서 분석법을 검토하였다. 분석컬럼은 C_{18}(5 μm, 250×2.0 mm I.D.)을 이용하였으며 이동상은 물과 ACN의 혼합액(40/60, v/v)으로 하였다. 이상의 조건에서 모든 *KR-V* 물질들은 용출시간 4~12분에 비교적 신속하게 잘 분리되었으며 정량한계는 15~30 ng/ml, 혈장중 회수율은 *KR-V* 2, 7 및 15를 제외하고는 85%(C.V. <10%) 이상으로 나타내었다. Ester 구조를 포함하고 있는 *KR-V* 2, 7 및 15는 혈장중에서 극히 불안정하여 유도체 개발의 분자설계상 제외시키는 것이 좋을 것으로 사료되었다. 결론적으로 본 HPLC-UV 검출법은 KR-V 시리즈 물질들의 약물동태연구를 위한 약물 분석법의 유효한 방법으로 검토되었다.

Key words : HIV, HPLC, *KR-V* series, rat plasma.