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Development and validation of column-switching high-performance liquid chromatographic methods for the determination of a potent AII receptor antagonist, TCV-116, and its metabolites in human serum and urine

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Abstract

Column-switching HPLC methods have been developed and validated for the determination of a new antihypertensive prodrug, TCV-116 (I), and its metabolites, CV-11974 (II) and CV-15959 (III), in human serum and urine. Initial sample cleanup was achieved by extracting the analytes into an organic solvent. After chromatographing on an ODS column with a mobile phase consisting of acetonitrile and an acidic phosphate buffer, the zone of the analyte's retention was heart-cut onto a second ODS column with a mobile phase of acetonitrile and a phosphate buffer at a higher pH. Complete separation of the analytes and the endogenous peaks was accomplished by the two-dimensional chromatography. Good precision and linearity of the calibration standards, as well as the inter-day and intra-day precision and accuracy of quality control samples, were achieved. The limit of quantitation (LOQ), using 0.5 ml of serum, was 2 ng/ml for I, 0.8 ng/ml for II, and 0.5 ng/ml for III. The LOQ for urine sample was 10 ng/ml for II and III. Stability of the analytes during storage, extraction, and chromatography processes was established. The results illustrate the versatile application of column switching to method development of multiple analytes in various biological matrices. The methods have been successfully used for the analyses of I and its metabolites in thousands of clinical samples to provide pharmacokinetic data.

1. Introduction

Angiotensin-converting enzyme inhibitors such as captopril and enalapril have been widely used for the treatment of hypertension [1]. The effects of nonpeptide angiotensin II (AII) receptor antagonists on the renin-angiotensin system have been investigated as a potential new class of antihypertensive agents [2–5]. TCV-116 (I), (±) - 1 - (cyclohexyloxycarbonyloxy)ethyl 2 - ethoxy - 1 - [[2′ - (1H - tetrazol - 5 - yl)biphenyl - 4 - yl]methyl] - 1H - benzimidazole - 7 - carboxylate, is a highly potent and long-acting AII receptor antagonist under drug development in Europe [5–7]. It acts as a prodrug, releasing the active metabolite CV-11974 (II) in vivo. II undergoes

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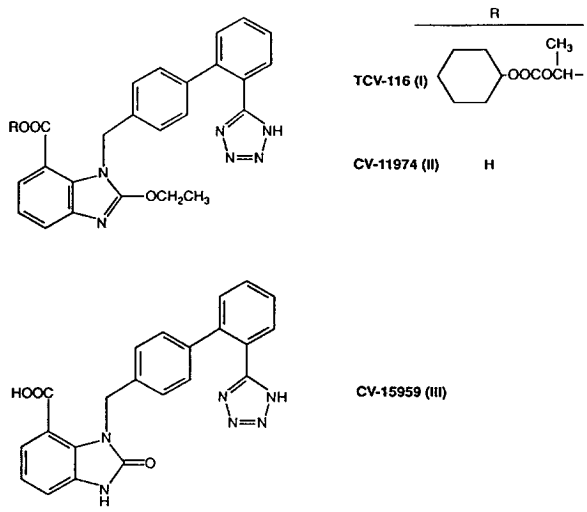

Fig. 1. Chemical structures of prodrug TCV-116 (I), metabolites CV-11974 (II) and CV-15959 (III).

further biotransformation to an inactive metabolite CV-15959 (III). The structures of these three compounds are shown in Fig. 1. In order to provide analytical support for clinical trials, sensitive methods to determine I and its metabolites in large numbers of serum and urine samples are required.

Analytical methods for nonpeptide AII receptor antagonists using conventional HPLC methods were reported [8,9]. Column-switching techniques for HPLC have been shown to be versatile and highly selective tools to separate various drugs from their metabolites and endogenous compounds in biological fluids [10]. The powerful resolution enabled sensitive UV detection of drug compounds at short-wavelength regions [11–15]. We exploit the flexibility of the column-switching technique to develop and validate HPLC methods for the quantitation of I, II, and III in human biological samples from clinical trials. Because I was rapidly converted into II and not detectable in urine samples during preliminary tests, the method for I in urine was not further investigated.

A simple liquid–liquid extraction step was used prior to the HPLC. A similar design of column-switching was used with slight variations of columns and mobile phases. A heart-cut fraction from column A was eluted to column B where a second mobile phase at a different pH provided a second dimensional separation of the compounds co-eluted from column A. I and II were determined by UV at 229 or 210 nm, III was determined by fluorescence with excitation at 285 nm and emission at 395 nm. The utilities of these methods have been demonstrated in thousands of assays on clinical samples.

2. Experimental

2.1. Materials and reagents

I, II and III were from Takeda Chemical Industries (Osaka, Japan). All inorganic chemicals of analytical grade were from Mallinckrodt (Paris, KT, USA). All organic solvents of HPLC grade were from Fisher (Fair Lawn, NJ, USA). Deionized water was purified by a NANOpure system from Barnstead. Control human serum was purchased from Worldwide Biologicals (Cincinnati, OH, USA). Control human urine was collected in house from healthy volunteers. Primary stock methanolic solutions of I, II, and III were prepared from separate weighings for standards and quality control samples (QCs). These solutions were stored at 4°C. Working standards were prepared fresh daily by spiking concentrated solutions into blank control serum and urine. Three levels of QCs at concentrations near the low, medium, and high working standards were prepared, aliquoted, and stored frozen at −20°C with the clinical samples to be analyzed.

2.2. Instrumentation

A schematic diagram of the HPLC system for analysis of I, II, and III in human serum and urine is shown in Fig. 2. Typical chromatographic conditions are summarized in Table 1. The pH of the mobile phase A and A′ was adjusted with 85% phosphoric acid. The pH of mobile phase B for I was adjusted with 1 *M* NaOH to 6.0. No pH adjustment is made for mobile phase B for the metabolites; the apparent pH was approximately 5. All chromatographic separations were done at

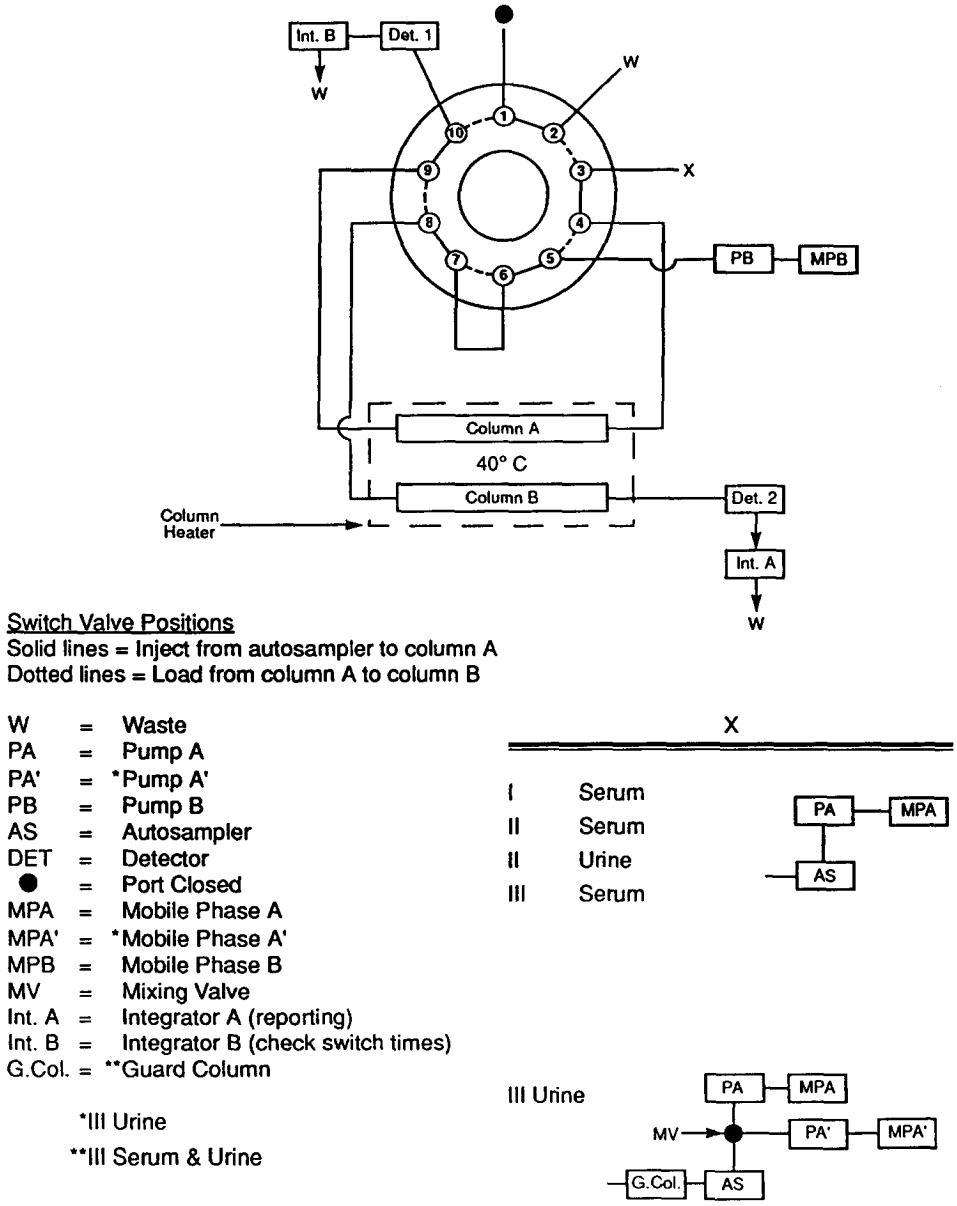

Fig. 2. Schematic diagram of the column-switching HPLC system.

40°C. The flow-rate was 1.0 ml/min except for II urine, the flow-rate for mobile phase A was 1.2 ml/min. Table 2 shows typical chromatographic column-switching time event programs. For I and II serum, the heart-cut fraction was made between the 1.5 and 2.0 min eluates from column A to column B. The corresponding heart-cut fraction of II urine was between 2.9 and 4.1 min, and that of III serum was between 8.8 and 9.6 min. For III urine, a strong mobile phase wash (A') was added to elute the late eluting peaks.

Because several HPLC systems were set up for the present study, the heart-cut window varied slightly on each system. Fig. 2 shows the position

Table 1
Chromatographic conditions

Matrix	Detection	Columns			Mobile phases (CH_3CN–20 mM KH_2PO_4, v/v)		
		Guard column	Column A	Column B	MPA′	MPA	MPB
I							
Serum	UV = 229 nm	N/A	YMC ODS 23 × 4.0 mm	YMC ODS 150 × 4.6 mm	N/A	60:40 pH 4.0	60:40 pH 6.0
II							
Serum	UV = 210 nm	N/A	[a]	[a]	N/A	32:68 pH 3.5	34:66
Urine	UV = 229 nm	N/A	[a]	[a]	N/A	27:73 pH 3.5	30:70
III							
Serum	Fluor: Ex = 285 nm Em = 395 nm	YMC ODS 23 × 4.0 mm	Inertsil ODS-2 150 × 4.6 mm	[a]	N/A	26:74 pH 3.0	26:74
Urine	[a]	Waters Novapak C_{18}	[a]	[a]	50:50 pH 3.0	[a]	21:79

[a] Same as the above.
Column suppliers: YMC, YMC, Wilmington, NC, USA; Inertsil, Keystone Scientific, Bellefonte, PA, USA; Waters, Millipore Corp., Milford, MA, USA.

of the switching valve when the sample was injected by the autosampler onto column A. The dotted lines indicate the position of the switching valve when the fraction containing the compound of interest was loaded from column A onto column B. The following HPLC instruments were used in various combinations for these methods: pumps: LDC 3200 and 3500, Beckman 100A, Waters 510; autosampler: Waters WISP 712 and 717, Shimadzu SIL-9A; detector (UV): LDC 3200; detectors (fluor): Shimadzu 530 (check column switch times), Shimadzu 535 and 551 (reporting); switching valve: Valco 10 port, electronically activated; column heaters: Timberline, Eppendorf; column switch/gradient controllers: Waters 680 controller, LDC 3500 programmable pump.

A linear regression, weighted $1/y$, was used to determine slopes, intercepts, and correlation coefficients, where y = the compound peak height.

Table 2
Column-switching time events (min)

		Inject	Load	Inject	Wash	Stop time
I	Serum	0	1.5	2.0	–	16.0
II	Serum	0	1.5	2.0	–	15.0
II	Urine	0	2.9	4.1	–	20.0
III	Serum	0	8.8	9.6	–	16.0
III	Urine	0	8.8	9.6	13.4	20.0
		100% MPA	100% MPB	100% MPB	100% MPA′	100% MPA

The resulting parameters were used to calculate concentrations: concentration = [peak height − (y-intercept)]/slope.

2.3. Extraction procedures

I in serum

To 0.5 ml serum, 0.5 ml of 0.2 *M* hydrochloric acid was added. After mixing, 5.0 ml of hexane–diethyl ether (1:1, v/v) was added to extract I by shaking for 15 min. An aliquot of 200 μl of 10% propylene glycol in methanol was added to the organic extract before evaporation under nitrogen. The residue was taken up in 200 μl of mobile phase A and 100 μl injected.

II in serum and urine

To 0.5 ml serum or urine, 0.5 ml of 0.2 *M* hydrochloric acid was added. After mixing, 5.0 ml of diethyl ether was added to extract II by shaking for 15 min. An aliquot of 100 μl of 10% propylene glycol in methanol was added to the organic extract before evaporation under nitrogen. The residue was taken up in 200 μl of mobile phase A and 100 μl injected for the serum sample and 50 μl for the urine sample.

III in serum

To 0.5 ml serum, 0.5 ml of 0.2 *M* hydrochloric acid was added. After mixing, 5.0 ml of ethyl acetate was added to extract III by shaking for 15 min. An aliquot of 100 μl of 10% propylene glycol in methanol was added to the organic extract before evaporation under nitrogen. The residue was taken up in 200 μl of mobile phase A and 100 μl injected onto the HPLC system.

III in urine

To 0.2 ml urine, 0.2 ml of 0.2 *M* hydrochloric acid was added. After mixing, 5.0 ml of diethyl ether was added to extract III by shaking for 15 min. An aliquot of 100 μl 10% propylene glycol in methanol was added to the organic extract before evaporation under nitrogen. The residue with propylene glycol was taken up in 1 ml of mobile phase A and 25 μl injected onto the HPLC system.

3. Results and discussion

3.1. HPLC separation

On a YMC ODS or an Inertsil ODS-2 column with a mobile phase consisting of acetonitrile and phosphate buffer, analytes were resolved from most of the endogenous peaks. With an increased pH of the mobile phase, the retention of II and III decreased dramatically. However, a single column could not provide a sensitive detection because of interference co-eluting with the analyte's peak. A conventional isocratic or gradient HPLC was unable to separate the analytes from interferences. We tried to clean up the samples by back-extraction procedures; however, the recoveries were much lower and inconsistent. A suitable internal standard (I.S.) was not available. A column-switching method was then developed to resolve the analytes from the interferences. The major advantage of the heart-cut column-switching method lies in its flexible cut of the selective fraction, providing powerful resolution of analytes from multiple interference peaks on two-dimensional chromatography. The high cost of chromatographic system set-up and the difficulty of incorporation of an I.S. remain the major drawbacks of this technique.

The fraction containing the compound of interest from column A was heart-cut onto column B. Mobile phase B was of a higher pH than mobile phase A. The analytes were completely resolved from the co-eluting endogenous peaks. A combination of a short (2.3 cm) column A and a long (15 cm) column B provided sufficient resolution for I and II while two long columns were necessary for III. For III, the peaks were sharpened on column B, resulting in an improved sensitivity. The peak sharpening may have been caused by a transient mobile phase pH gradient formed on column B after switching. UV detection gave enough sensitivity for I and II. For the more polar III, fluorescence detection offered greater selectivity than UV.

For III urine assay, a stronger mobile phase A′ was used to elute the late peaks from column A. This wash extended the life of column A and avoided diverting interferences onto column B.

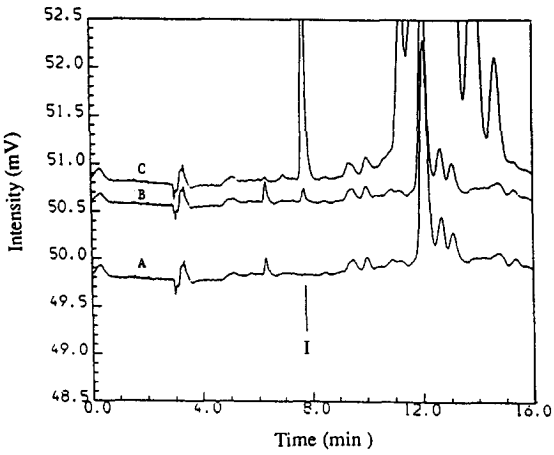

Fig. 3. Typical chromatograms of extracted blank control serum (A), standard at 2.0 ng/ml (B), and QC at 50 ng/ml (C) of I.

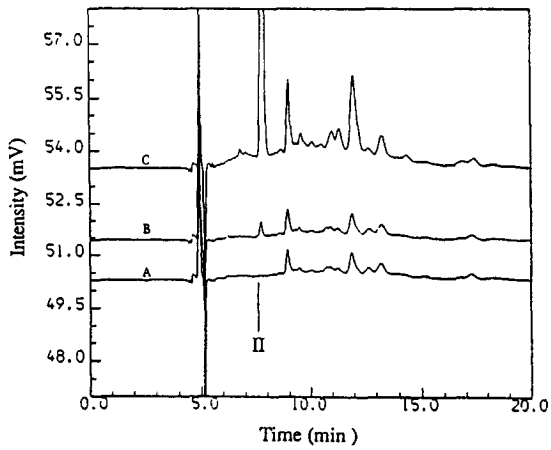

Fig. 5. Typical chromatograms of extracted blank control urine (A), standard at 10 ng/ml (B), and QC at 750 ng/ml (C) of II.

The wash was not needed for the other assays because late-eluting interferences were not observed. The III heart-cut window was monitored on column A to allow necessary adjustment. A wide window lead to more interferences. For the analysis of I and II this monitoring was not required because of its constant retention time on a short column.

Figs. 3–7 show chromatograms of extracted blank control serum and urine, standards at LOQ and QCs. At least six blank control serums and urine were screened, and no interferences were observed. The analytical columns were stable for at least several hundred injections under the chromatographic conditions. Several batches of analytical columns were used throughout the studies and no batch-to-batch variabilities were observed.

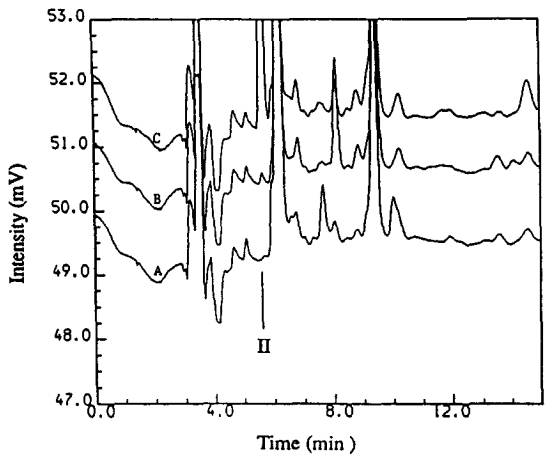

Fig. 4. Typical chromatograms of extracted blank control serum (A), standard at 0.8 ng/ml (B), and QC at 75 ng/ml (C) of II.

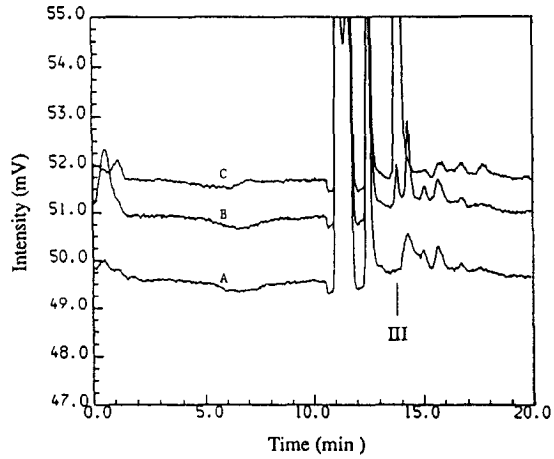

Fig. 6. Typical chromatograms of extracted blank control serum (A), standard at 0.5 ng/ml (B), and QC at 30 ng/ml (C) of III.

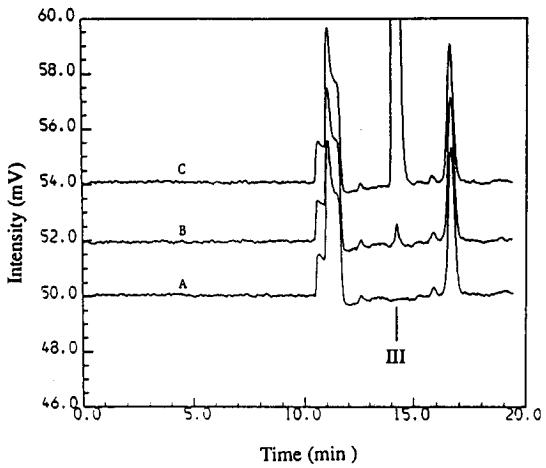

Fig. 7. Typical chromatograms of extracted blank control urine (A), standard at 10 ng/ml (B), and QC at 2400 ng/ml (C) of III.

3.2. Extraction

Because our approach was to use a heart-cut column-switching technique to separate the analytes from the endogenous compounds, we avoided developing complicated, multiple extraction procedures for analytes in different matrices. A simple one-step extraction was used to clean up the biological samples prior to chromatography. After acidifying the samples, the analyte was extracted into an organic solvent. Propylene glycol was added to the extract prior to evaporation to keep the extract from complete dryness. This addition resulted in better recovery and more consistent data for II. Overall recovery was improved from 50% to 74%, and consistency was improved from a coefficients of variance (C.V.) range of 4.2–15.3% without addition to 4.5–8.4% with the addition. Table 3 summarizes the recoveries of I, II and III from human biological matrices. Almost complete recoveries for I and III were obtained. For II, the recoveries were about 70% from serum and 80% from urine. All recoveries were consistent throughout the entire standard concentration ranges. Although an I.S. was not used in the method, the excellent consistency in recovery eliminated this need.

3.3. Validation performance

Table 4 shows the linearity and precision data of the validation curves. It should be noted that the standard curve range and LOQ were designed to suit the analysis of each analyte in serum or urine. LOQ was defined as the lowest standard level which meets the acceptance criteria in accuracy and precision of ≤15% bias and C.V. from five or more determinations [16]. Very good linearity was observed for all of the standard curves. Table 5 shows the inter-day and intra-day accuracy and precision of the QCs. The accuracy and precision data show that the methods were consistent and reliable with low relative error (R.E.) and C.V. for standards and QCs over

Table 3
Recovery of I, II, and III from biological matrices

In serum (ng/ml)	I			II			III		
	2.0	20	60	0.8	10	100	0.5	10	50
Mean recovery (%)	85.3	91.5	91.3	67.5	69.5	69.8	102	90.8	89.6
C.V. (%)	3.5	2.9	2.9	5.5	1.5	3.6	4.7	2.4	1.9
In urine (ng/ml)	I			II			III		
	NP	NP	NP	10	100	1000	10	250	5000
Mean recovery (%)				79.4	81.5	81.8	104	95.9	96.5
C.V. (%)				2.9	2.0	1.8	7.1	3.6	2.7

NP = not performed, $n = 6$.

Table 4
Precision and linearity of standards

In serum											
I (ng/ml)	2.0	3.0	5.0	7.5	10	20	30	40	60		*r*
Mean (ng/ml)	1.96	3.01	4.93	7.56	10.2	20.4	30.3	39.3	59.9		0.9998
C.V. (%)	3.5	2.5	1.3	1.0	4.2	1.8	0.7	0.7	0.8		0.02
R.E. (%)	−2.1	+0.3	−1.5	+0.8	+2.2	+1.8	+1.1	−1.7	−0.1		
n	5	5	5	5	5	5	5	5	5		5
II (ng/ml)	0.8	1.0	2.0	3.0	5.0	10	30	50	80	100	*r*
Mean (ng/ml)	0.74	1.01	1.98	2.94	5.01	10.1	29.4	49.6	80.3	103	0.9998
C.V. (%)	10.8	8.2	9.1	3.1	4.2	3.2	4.4	1.5	1.0	2.5	0.23
R.E. (%)	−7.4	+1.3	−1.2	−2.0	+0.2	+0.9	−2.0	−0.8	+0.4	+3.2	
n	5	5	5	5	5	5	5	5	5	5	5
III (ng/ml)	0.5	1.0	2.0	4.0	5.0	10	20	40	50		*r*
Mean (ng/ml)	0.52	0.99	1.96	3.98	4.97	10.2	19.8	39.9	50.2		0.9998
C.V. (%)	5.3	2.7	2.4	2.9	2.4	2.3	3.0	1.6	1.4		0.01
R.E. (%)	+4.2	−0.6	−2.2	−0.4	−0.6	+2.0	−1.2	−0.2	+0.5		
n	6	6	6	6	6	6	6	6	6		6
In urine											
II (ng/ml)	10	20	60	100	200	400	800	1000			*r*
Mean (ng/ml)	9.5	20.5	61.1	99.4	201	408	814	979			0.9996
C.V. (%)	1.5	2.2	2.3	2.7	2.1	1.5	1.7	2.0			0.00
R.E. (%)	−5.2	+2.5	+1.8	−0.6	+0.7	+1.9	+1.7	−2.1			
n	5	5	5	5	5	5	5	5			5
III (ng/ml)	10	25	50	100	250	1000	2500	5000			*r*
Mean (ng/ml)	10.1	24.5	50.2	99.8	251	1010	2530	4960			0.9998
C.V. (%)	6.0	3.0	1.9	2.3	2.3	3.9	2.2	1.4			0.03
R.E. (%)	+0.7	−2.2	+0.4	−0.2	−0.2	+1.3	+1.1	−0.7			
n	5	5	5	5	5	5	5	5			5

the entire concentration range. The LOQ for 0.5 ml serum sample was 2.0, 0.8 and 0.5 ng/ml for I, II, and III, respectively. The LOQ for urine sample was 10 ng/ml for II and III. The sensitivity of the method is better than the conventional chromatographic methods for other AII receptor antagonists reported in the literature. Other imidazole AII receptor antagonists using conventional HPLC methods had LOQ 2–5 ng/ml [8,9].

3.4. Stability

Stabilities of processing (freeze–thaw cycles and benchtop), chromatography (on-system and re-injection), and sample storage were established. The data for I, II, and III were presented in Tables 6, 7, and 8, respectively. To mimic the possible freezing and thawing conditions of clinical samples, QCs were subjected to various cycles of freezing and thawing and then analyzed. The values of QCs after multiple freeze–thaw cycles were comparable to those of one cycle for the analytes in human serum or urine. All results of stability tests of benchtop, sample storage, on-system, and reinjection were comparable to the controls. The results show that no degradation of the analytes occurs during sample storage, chromatography, and extraction processes.

Table 5
Precision and accuracy of quality controls

	Inter-day						Intra-day					
	In serum			In urine			In serum			In urine		
I (ng/ml)	6.0	15	50	NP			6.0	15	50	NP		
Mean (ng/ml)	5.58	14.4	47.9				5.64	14.4	48.9			
C.V. %	2.3	3.2	4.1				3.5	3.7	2.4			
R.E. %	−6.9	−4.2	−4.2				−6.1	−3.9	−2.2			
n	30	30	30				6	6	6			
II (ng/ml)	2.4	15	75	30	160	750	2.4	15	75	30	160	750
Mean (ng/ml)	2.45	15.0	76.0	30.2	158	749	2.50	15.8	79.3	29.9	160	740
C.V. %	9.4	12.6	7.3	4.7	4.2	6.0	2.6	1.1	0.4	2.2	3.2	2.2
R.E. %	+2.0	0	+1.3	+0.6	−1.4	−0.1	+4.3	+5.4	+5.7	−0.4	+0.1	−1.3
n	30	30	30	31	31	31	6	6	6	6	6	6
III (ng/ml)	1.5	15	30	30	180	2400	1.5	15	30	30	180	2400
Mean (ng/ml)	1.48	15.3	30.1	30.0	180	2520	1.42	14.7	29.6	30.9	180	2480
C.V. %	5.6	8.6	5.2	4.2	3.9	3.6	3.8	4.9	5.8	1.2	1.0	2.5
R.E. %	−1.6	+2.3	+0.2	0	0	+5.2	−5.6	−1.9	−1.4	+2.9	0	+3.3
n	36	36	35	30	30	30	6	6	6	6	6	6

NP = Not performed.

Table 6
Stability of I quality controls in human serum

	I (ng/ml)		
	6.0	15	50
Freeze–thaw (*n* = 6)			
1st cycle	5.64 (3.5)	14.4 (3.7)	48.9 (2.4)
2nd cycle	5.72 (3.3)	14.1 (3.5)	49.5 (6.7)
As % of 1st cycle	101	97.9	101
3rd cycle	5.55 (4.1)	14.4 (3.9)	48.4 (4.2)
As % of 1st cycle	98	100	99
Bench top (*n* = 6)			
Normal	5.64 (3.5)	14.4 (3.7)	48.9 (2.4)
3 h	5.61 (5.7)	14.3 (4.2)	49.3 (3.1)
As % of normal	100	99	101
On system			
Initial (*n* = 3)	5.01 (0)	13.5 (0)	45.9 (0.3)
31 h (*n* = 4)	5.47 (8.2)	14.4 (5.1)	48.3 (5.9)
As % of of initial	109	107	105
Sample storage			
Initial (*n* = 6)	5.73 (1.4)	14.7 (1.0)	49.6 (1.4)
43 weeks (*n* = 5)	5.89 (3.4)	17.0 (14.4)	55.1 (4.4)
As % of initial	103	116	111

Table 7
Stability of II quality controls in human serum and urine

	In serum (ng/ml)			In urine (ng/ml)		
	2.4	15	75	30	160	750
Freeze–thaw ($n = 6$)						
1st cycle	2.50 (2.6)	15.8 (1.1)	79.3 (0.4)	28.8 (3.0)	151 (4.1)	717 (3.8)
2nd cycle	2.50 (2.2)	15.8 (2.4)	78.5 (0.8)	27.6 (3.8)	147 (2.3)	711 (1.7)
As % of 1st cycle	100	100	99	96	97	99
3rd cycle	2.52 (0.9)	15.6 (1.6)	79.2 (2.1)	26.7 (6.1)	146 (3.2)	702 (2.8)
As % of 1st cycle	101	99	100	93	97	98
Bench top ($n = 6$)						
Normal	2.18 (6.2)	14.6 (2.5)	80.2 (5.6)	31.3 (2.5)	162 (1.9)	756 (3.4)
2 h[a]	2.31 (8.3)	15.8 (9.3)	77.7 (6.5)	30.3 (2.0)	154 (4.0)	749 (3.1)
As % of normal	106	108	97	97	95	99
On system ($n = 3$)						
Initial	2.60 (1.5)	15.7 (1.8)	78.8 (0.9)	31.0 (0.9)	154 (0.4)	759 (0.5)
24 h[b]	2.52 (1.0)	15.7 (9.1)	78.3 (0.3)	31.5 (2.0)[c]	163 (7.8)[c]	796 (8.2)[c]
As % of initial	97	100	99	102	106	105
Reinjection ($n = 6$)						
Initial	NP	NP	NP	29.9 (2.2)	160 (3.2)	740 (2.2)
10 h				33.2 (2.6)	168 (5.5)	771 (4.1)
As % of initial				111	105	104
Sample storage						
Initial	2.34 (3.5)	14.4 (5.4)	76.0 (7.2)	30.8 (1.8)	162 (3.2)	757 (0.8)
n	6	6	6	6	6	6
8 months[d]	2.48 (7.7)	13.9 (7.1)	73.6 (4.6)	33.5 (4.3)	184 (1.4)	835 (2.5)
n	2	2	2	6	6	6
As % of initial	106	97	97	109	114	110

[a] 4.5 h for II in urine.
[b] 54 h for II in urine.
[c] $n = 5$.
[d] 11 weeks for II in urine.
NP = Not performed due to limited sample volume. C.V.s (%) are indicated in parentheses.

4. Application

The column-switching HPLC methods developed here were used to study the pharmacokinetic profiles of the prodrug I and its metabolites. The drug concentration versus time profiles of II and III in serum from a volunteer dosed orally with a 16-mg tablet of I are shown in Fig. 8. Fig. 9 shows the amount of II and III excreted in urine. In vivo, I was completely and rapidly transformed to II and was not detectable. II was further metabolized to III. Because I was not found in serum clinical samples, the analysis of I in urine was not carried out.

5. Conclusions

Heart-cut column-switching methods were developed for the analyses of I and its metabolites, II and III, in human serum and urine. With the combination of coupled columns and different mobile phase compositions, it has been demonstrated that this technique was very suitable for separating the analytes from multiple matrix interferences. This method has been successfully used in our laboratory for the analysis of large numbers of samples in clinical trials. The method is rugged and reproducible through the use of multiple systems, recoveries are consistent with-

Table 8
Stability of III quality controls in human serum and urine

	In serum (ng/ml)			In urine (ng/ml)		
	1.50	15	30	30	180	2400
Freeze–thaw ($n = 6$)						
1st cycle	1.42 (3.8)	14.7 (4.9)	29.6 (5.8)	31.0 (3.0)	189 (2.6)	2670 (2.2)
2nd cycle	1.38 (1.9)	14.8 (3.5)	31.8 (3.8)	31.9 (1.4)	191 (1.8)	2680 (2.3)
As % of 1st cycle	97	101	107	103	101	100
3rd cycle	1.80 (11)	15.6 (6.2)	32.5 (8.8)	31.6 (3.3)	193 (2.0)	2680 (1.8)
As % of 1st cycle	127	106	110	102	102	100
Bench top ($n = 6$)						
Normal	1.58 (4.1)	16.4 (16)	30.0 (3.3)	28.8 (4.0)	175 (2.2)	2500 (2.2)
4 h[a]	1.58 (7.8)	15.9 (7.8)	31.6 (5.5)	29.4 (4.9)	172 (4.6)[b]	2460 (3.6)
As % of normal	100	97	105	102	98	98
On system ($n = 3$)						
Initial	1.35 (3.3)	13.6 (3.2)	30.3 (0.0)	27.7 (3.7)	170 (2.6)	2410 (5.2)
21 h[c]	1.64 (7.3)	15.0 (3.1)	33.0 (9.5)	30.9 (2.6)	190 (0.3)	2660 (1.2)
As % of initial	121	110	109	112	112	110
Reinjection ($n = 6$)						
Initial	NP	NP	NP	30.9 (1.5)	181 (1.0)	2508 (2.1)
29 h				31.7 (2.5)	185 (0.3)	2560 (1.0)
As % of initial				103	102	102
Sample storage ($n = 6$)						
Initial	1.42 (3.8)	14.7 (4.9)	29.6 (5.8)	29.1 (2.7)	173 (1.9)	2420 (1.6)
27 weeks[d]	1.33 (1.8)	13.7 (2.4)	28.2 (2.5)	27.5 (2.3)	171 (3.9)[b]	2290 (5.7)
As % of initial	94	93	95	95	99	95

[a] 2 h for III in urine.
[b] $n = 5$.
[c] 25 h for III in urine.
[d] 8 weeks for III in urine.
NP = Not performed due to limited sample volume. C.V.s (%) are indicated in parentheses.

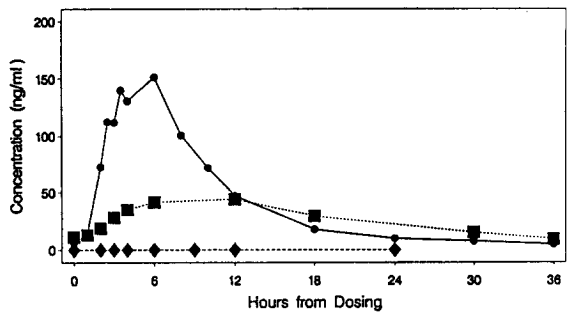

Fig. 8. Pharmacokinetics profiles of I, II and III from serum samples of a participant receiving a single oral dose of 16 mg I. Symbols: ◆ = I, ● = II, ■ = III.

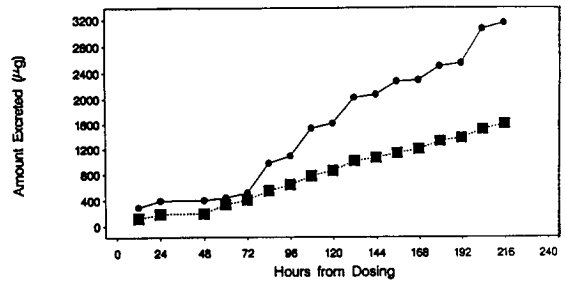

Fig. 9. Urine excretion of II and III over time from a participant receiving multiple oral doses of 16 mg I. Symbols: ● = II, ■ = III.

out the need of an I.S. Stabilities of I, II, and III during storage, extraction, and chromatography have been established to assure sample and

processing stability during the time span of drug development for this compound.

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