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Quantitation of *N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyloxy-4-methoxybenzamide and 4-amino-3,5-dichloropyridine in rat and mouse plasma by LC/MS/MS

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Abstract

The metabolism of *N*-(3,5-dichloropyrid-4-yl)-3-cyclopentyloxy-4-methoxybenzamide (RP73401), a phosphodiesterase IV (PDE IV) inhibitor is extensive (unpublished); however, until recently, studies for this compound did not report 4-amino-3,5-dichloropyridine (ADCP) as a metabolite either in vitro or in vivo. This prompted a reinvestigation into the metabolism of RP73401 in rats and mice using mass spectrometry. The results of the reinvestigation confirmed that 4-amino-3,5-dichloropyridine was formed via the metabolism of RP73401 both in vitro and in vivo. In order to further investigate RP73401 hydrolysis in vivo, a liquid chromatography/mass spectrometry assay was developed and validated for the simultaneous determination of RP73401 and ADCP in rat and mouse plasma. The method used Waters Oasis HLB brand solid phase extraction cartridges to isolate the analytes (RP73401 and ADCP) and internal standard from the plasma. HPLC chromatographic separation was achieved using a Zorbax SB C18 HPLC column and detection was accomplished using positive ion atmospheric pressure chemical ionization tandem mass spectroscopy in multiple reaction monitoring (MRM) mode. The assay was developed and validated over the range of 0.5–100 ng ml^{-1} for RP73401 and 5–500 ng ml^{-1} for ADCP using 0.050 ml of plasma. The assay proved to be sensitive, accurate, precise and specific for RP73401 and ADCP. Intraday and interday quality control results routinely showed accuracy and precision to be within $\pm 20\%$. This LC/MS/MS method was subsequently employed to investigate the hydrolysis of RP73401 in the rat and mouse, and determine the effects of tri-o-tolyl phosphate (TOTP, a carboxylesterase inhibitor) preadministration on the hydrolysis reaction in the rat.

Keywords: Atmospheric pressure chemical ionization; Tri-o-tolyl phosphate; 4-Amino-3,5-dichloropyridine; Quantitation

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1. Introduction

RP73401 (*N*-(3,5-dichloropyrid-4-yl)-3-cylcopentyloxy-4-methoxybenzamide) [1,2] (Fig. 1), a phosphodiesterase IV inhibitor [3–5], has been in development for the prophylactic treatment of airway inflammation and bronchoconstriction. The in vitro and in vivo metabolism of this compound is extensive (unpublished), however previous metabolism studies did not report 4-amino-3,5-dichloropyridine as a metabolite of RP73401 (Fig. 1). While performing safety assessment investigations, a mass spectrometry assay identified that 4-amino-3,5-dichloropyridine, a product of RP73401 hydrolysis, is formed in vitro and in vivo. The discovery of this new metabolite, therefore, prompted further investigation.

Previously, no assay for the determination of ADCP or the simultaneous determination of RP73401 and ADCP in biological matrices had been reported. Liquid Chromatography/Tandem Mass Spectrometry (LC/MS/MS) with atmospheric pressure chemical ionization (APCI) is a common technique used for the determination of pharmaceuticals in biological matrices [6–9]. Due to the high sensitivity of LC/MS/MS, a small sample size of 0.050 ml is sufficient to obtain lower limits of quantitation (LOQ) in the low ng ml^{-1} range. Consequently, an LC/MS/MS assay was developed and validated to quantitate RP73401 and ADCP in rat and mouse plasma. Furthermore, the method was utilized to generate toxicokinetic data which compared the hydrolysis of RP73401 in the rat and mouse and determined the effects of tri-o-tolyl phosphate [10,11] (TOTP, a carboxylesterase inhibitor) on the hydrolysis of RP73401 to yield ADCP in the rat.

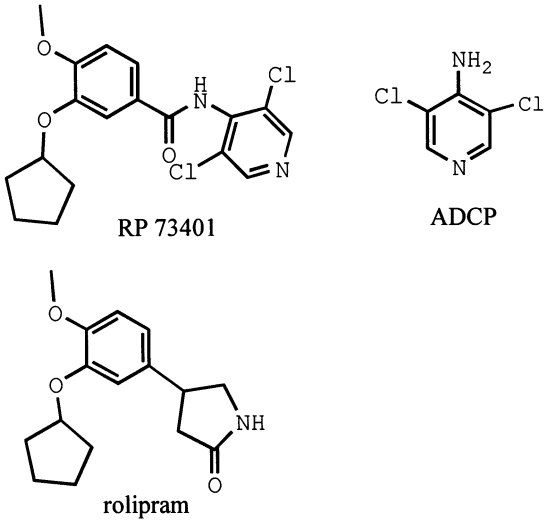

Fig. 1. Chemical structures of RP73401, ADCP and rolipram.

2. Materials and methods

2.1. Chemicals and materials

Compounds RP73401, ADCP and rolipram (internal standard) were synthesized at Aventis Pharma (Collegeville, PA, USA). Control rat and mouse plasma was obtained from Lampire Biological Laboratories (Pipersville, PA, USA). HPLC grade MeOH, water and ammonium acetate were obtained from EM Science (Gibbstown, NJ, USA). Spectrophotometric grade trifluoroacetic acid was obtained from Aldrich Chemical Company (Milwaukee, WI, USA). Waters Oasis HLB extraction cartridges (30 mg) were obtained from Waters Corp. (Milford, MA, USA). The TurboVap analytical evaporator was obtained from Zymark (Hopkinton, MA, USA).

2.2. LC/MS equipment and analytical conditions

A Hitachi HPLC system consisting of an L6000 pump, an L6200 pump (controller) and a AS 4000 autosampler was used for HPLC analyses. The chromatographic system consisted of a Zorbax SB C18 (4.6 mm id × 15 cm, 5 μm) column and an isocratic mobile phase of MeOH-aqueous 50 mM NH_4OAc (70:30, v/v) which was delivered to the mass spectrometer at 0.8 ml min^{-1}. The autosampler was programmed to inject samples every 10 min. Mass spectrometric detection was performed on a Sciex API III plus instrument operating in positive ion APCI mode. The heated nebulizer was set at 450°C with a discharge ionization of 5 μA. The orifice potential was set at 60 V and the collision gas thickness (Ar) was set at approximately 240×10^{12} atom cm^{-2}. Nitrogen was used

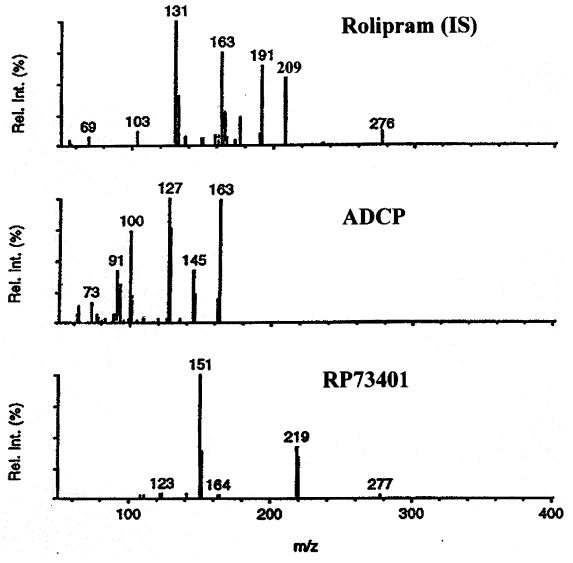

Fig. 2. Product ion spectra of rolipram, ADCP and RP73401.

as the auxiliary and nebulizer gas and was set at 2 l min^{-1} and 65 psi, respectively. Quantitation was performed using multiple reaction monitoring (MRM) of the transitions RP73401 m/z 381 → 151, ADCP m/z 163 → 127 and rolipram m/z 276 → 131 with a scan time of 0.62 scans s^{-1} and a dwell time of 400 ms. The product ion spectra of RP73401, ADCP and internal standard are shown in Fig. 2. Mass calibration, data acquisition, mass spectral representation and post-acquisition quantitation analyses were performed with Sciex software: Tune 2.5, RAD 2.6, MacSpec 3.3, and MacQuan 1.3 using a MacIntosh Quadra 900 computer.

2.3. Standard stock and working solutions

Stock standards of RP73401 and ADCP were prepared in MeOH (1 mg ml^{-1}). These solutions were diluted with MeOH to give a series of working solutions used to prepare plasma pools. Two separate weighings were used to prepare the standard curve stock solution and the quality control stock solution. The internal standard spiking solution was prepared by diluting a rolipram stock solution with MeOH to give a 40 ng ml^{-1} spiking solution. All stock solutions and working solutions were stored at −20°C until use.

2.4. Rat and mouse plasma pools

Rat and mouse plasma pools were prepared by adding the appropriate volume of working solution to the appropriate volume of blank rat or mouse plasma. A series of calibration curve pools were prepared at final concentrations of 0.5/5, 1/10, 5/50, 10/100, 25/250, 50/500, 100/0 ng ml^{-1} RP73401/ADCP, respectively. Similarly, quality control plasma pools were prepared at final concentrations of 0.5/5, 1/10, 5/50 and 50/500 ng ml^{-1} RP73401/ADCP, respectively. RP73401 was found to be stable in rat and mouse plasma for 24 h at room temperature and for at least three freeze/thaw cycles. All plasma pools were aliquoted and then stored at −20°C until use.

2.5. Extraction procedure

The following procedure was used to extract standards, blanks, controls and unknown samples: 50 μl of standard, control, blank plasma or unknown sample was added to culture tube followed by 0.5 ml of water and 50 μl of internal standard spiking solution (40 ng ml^{-1}). The above sample was applied to a preconditioned Oasis™ SPE column, eluted, then washed with water (1 ml). The analytes and internal standard were eluted from the column with methanol (1 ml). Prior to drying the sample, 250 μl of 1% trifluoroacetic acid–methanol (1/100, v/v) was added to the eluate. The samples were concentrated to dryness on the TurboVap then reconstituted with 100 μl of methanol–water (70/30, v/v). A total of 90 μl of reconstituted sample was injected onto the LC/MS/MS system.

2.6. Validation procedures

The assay was validated over five analytical batches (three from rat and two from mice). The validation examined calibrator plasma pools with levels of 0.5/5, 1/10, 5/50, 10/100, 25/250, 50/500, 100/0 ng ml^{-1} RP73401/ADCP, respectively. The 100/0 ng ml^{-1} RP73401/ADCP pool was chosen to verify the purity of the RP73401 lot and to confirm the hydrolytic stability of RP73401 to the extraction and analysis procedures. Control sam-

ple plasma pools at levels of 0.5/5, 1/10, 5/50, 50/500 ng ml^{-1} RP73401/ADCP, respectively were assayed in replicate to ascertain both intraday and interday precision and accuracy. Separate lots of blank plasma (rat and mouse) were assayed to assure specificity. A dilution sample (1/2 the highest quality control) was assayed in replicate to access dilution integrity. Finally, standards which had not been extracted were assayed to access analyte(s) and internal standard recovery from plasma. Since the rat and mouse standard curve parameters (slope, intercept) were indistinguishable, the interday precision and accuracy statistics for the assay were determined by combining data from the rat and mouse.

2.7. In vivo experiments

2.7.1. 'Hydrolysis of RP73401 in rats and mice', oral administration of RP73401 or ADCP

The rats and mice were separated into three drug dosing groups for the rat and four drug dosing groups for the mouse. For each species there was one control group. For the rat, one group received 250 mg kg^{-1} of RP73401 orally and the other two groups were orally dosed with 0.1 or 1.0 mg kg^{-1} of ADCP, respectively. For the mouse, two groups were dosed orally 250 or 2000 mg kg^{-1} of RP73401. The remaining two groups of mice were administered either 3 or 10 mg kg^{-1} of ADCP, respectively. Blood samples, collected in heparinized tubes, were obtained from anesthetized rats and mice via the orbital venous plexus for rat and mouse at 0.5 h/1 h, from the abdominal aorta for rat at 2 h/4 h and by cardiac puncture for the mouse at 2 h/4 h. Blood samples were centrifuged and the plasma decanted and frozen at -20°C prior to analysis.

2.7.2. 'Effects of Tri-o-tolyl phosphate on RP73401 hydrolysis in the rat'

In a separate experiment, rats were separated into six drug dosing and two control groups. In one arm of the study, rats were treated orally with 300 mg kg^{-1} of TOTP approximately 12 h prior to the oral administration of RP73401 (75 or 125 mg kg^{-1}) or ADCP (1 mg kg^{-1}). In the second arm of the study, rats received RP73401 (75 or 125 mg kg^{-1}) or ADCP (1 mg kg^{-1}) without TOTP pretreatment. Blood samples were collected in heparinized tubes via the orbital venous plexus from anesthetized rats at 1, 4 and 24 h for one set of animals and 2, 8 and 24 h for a second set of animals. Samples were centrifuged and the plasma was decanted and frozen at -20°C prior to analysis.

3. Results and discussion

The goal of this work was to develop and validate a rugged, reproducible and sensitive assay for the quantitation of RP73401 and ADCP in rat and mouse plasma which would be suitable for determining the exposure of these compounds in animals involved in toxicology studies. The LC/MS/MS technique was chosen due to the high specificity and sensitivity requirements of the assay.

3.1. Linearity, specificity and sensitivity

The linearity of the method was evaluated by extracting calibration curves in rat and mouse plasma in five batches on 5 separate days. Calibrators were assayed in ascending order at the beginning of each batch. The data indicate that the assay is linear from 0.500 to 100 ng ml^{-1} for RP73401 and from 5 to 500 ng ml^{-1} for ADCP. The coefficients of determination (r^2) were >0.98 for both RP73401 and ADCP for all batches.

The specificity of the assay was determined by the analysis of drug free rat and mouse plasma controls. Two lots of rat plasma and one lot of mouse plasma were assayed. Analysis indicated that, at the retention times of interest, no interfering endogenous peaks were present. A typical chromatogram for drug free plasma is shown in Fig. 3.

Quality control samples were assayed at the lowest limit of quantitation (LLOQ) in replicate (five replicates for intraday, two replicates for interday) in both rat and mouse plasma (0.5 ng ml^{-1} for RP73401 and 5 ng ml^{-1} for ADCP). The intraday precision and accuracy for RP73401 was $<7.9\%$ and $<6.4\%$ (absolute value), while for

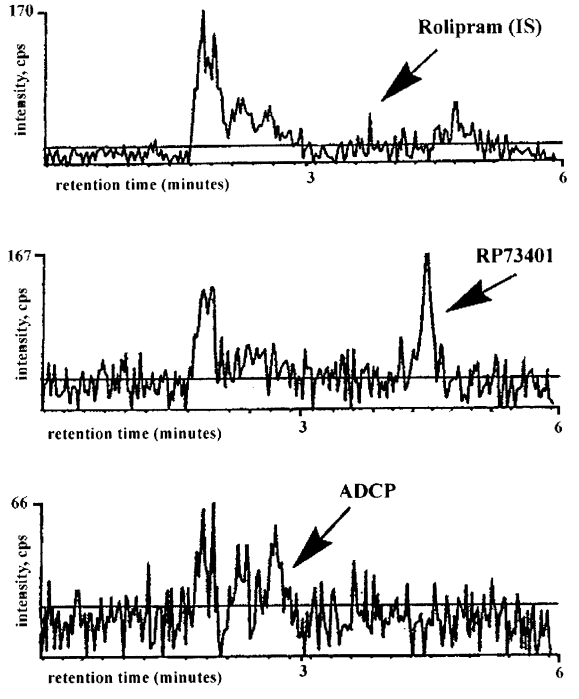

Fig. 3. Typical MRM chromatograms of drug free plasma (rat).

ADCP the intraday precision and accuracy was < 8.1% and < 8.0% (absolute value).

The mean interday precision and accuracy for RP73401 was 8.2 and 0.2% (absolute value), while for ADCP the mean interday precision and accuracy was 8.8 and 2.6% (absolute value). Intraday control results for accuracy and precision for rat and mouse are shown in Table 1. Interday accuracy and precision, presented by combining rat and mouse data for all five batches, are shown in Table 2. A typical chromatogram at the LLOQ is shown in Fig. 4.

3.2. Precision and accuracy of controls

Quality control samples were assayed in replicate (five replicates for intraday and two replicates for interday) at three levels for RP73401 (1, 5, 50 ng ml^{-1}) and ADCP (10, 50, 500 ml^{-1}). The intraday precision and accuracy for RP73401 was < 8.9% and < 15.5% (absolute value). The mean interday precision and accuracy for RP73401 was < 10.0% and < 3.0% (absolute value). The intraday precision and accuracy for ADCP was < 18.5% and < 12.9% (absolute value). The mean interday precision and accuracy for ADCP was < 13.6% and < 8.6% (absolute value). Results are shown in Tables 1 and 2.

3.3. Dilution and recovery

Dilution integrity was demonstrated by assaying a dilution control (one half dilution of 50/500

Table 1
IntraDay QC results for rat and mouse

		Validation sample level (ng ml^{-1})							
		RP7304				ADCP			
Species	Parameter	0.50	1.00	5.00	50.00	5.00	10.00	50.00	500.00
Rat	*n*	5	4	5	5	5	5	5	5
	Average (ng ml^{-1})	0.49	0.93	4.73	47.60	4.65	10.56	44.69	439.70
	SD (ng ml^{-1})	0.04	0.08	0.15	3.49	0.38	0.35	2.42	57.54
	Accuracy (%RE)	−1.2	−6.8	−5.4	−4.8	−7	5.6	−10.6	−12.1
	Precision (%RSD)	7.9	8.9	3.2	7.3	8.1	3.3	5.4	13.1
Mouse	*n*	5	5	5	5	5	5	5	5
	Average (ng ml^{-1})	0.53	1.03	5.78	51.96	4.60	10.12	52.86	435.74
	SD (ng ml^{-1})	0.04	0.05	0.32	1.96	0.20	1.23	9.77	35.11
	Accuracy (%RE)	6.4	3	15.5	3.9	−8	1.2	5.7	−12.9
	Precision (%RSD)	7	4.5	5.6	3.8	4.3	12.1	18.5	8.1

Table 2
Interday day QC results

		Validation sample level (ng ml^{-1})							
		RP 73401				ADCP			
Species	Parameter	0.50	1.00	5.00	50.00	5.00	10.00	50.00	500.00
Rat/mouse	*N*	19	15	16	16	19	16	16	16
	Average (ng ml^{-1})	0.50	0.99	5.15	49.92	4.87	10.67	49.30	456.79
	SD (ng ml^{-1})	0.04	0.10	0.52	3.08	0.43	0.87	6.68	47.08
	Accuracy (%RE)	0.2	−1.1	3	−0.2	−2.6	6.7	−1.4	−8.6
	Precision (%RSD)	8.2	9.8	10	6.2	8.8	8.2	13.6	10.3

ng ml^{-1} RP73401/ADCP) in replicate (5). The mean precision and accuracy for RP73401 and ADCP were both $< 10\%$ (absolute value). The recovery of analytes and internal standard was determined by comparing the peak area responses from extracted samples to equivalent absolute standards which had not been extracted. Recovery samples were assayed in triplicate. The mean recovery of RP73401 was found to be 74 and 53% for ADCP. The addition of TFA to the eluate prior to drying the samples was critical to recover ADCP. The recovery of the internal standard at the 2 ng ml level was approximately 79%.

3.4. In Vivo results

3.4.1. Rat and mouse results

Mean ($\pm$ S.D.) plasma concentration data and AUC's are presented in Table 3, and Fig. 5. Qualitatively, ADCP was confirmed in rat and mouse plasma from animals which had been administered a single, oral dose of RP73401. However, distinct differences in the relative amounts of RP73401 and ADCP in plasma were observed for different dose groups, as well as for the rat and mouse. A quantifiable amount of ADCP was observed in the rat following both RP73401 and ADCP administration. However, although ADCP was detectable in the plasma of mice receiving ADCP and RP73401, plasma concentrations of ADCP were below the limit of quantitation in mice receiving oral RP73401. The AUC results also indicate that ADCP has better bioavailability in the rat than in the mouse.

3.4.2. Effect of TOTP on RP73401 hydrolysis

The results from TOTP pretreatment experiments are shown in Fig. 6. Distinct differences in relative amounts of ADCP were observed between rats with and without TOTP pretreatment followed by an oral dose of RP73401. Apparently, pretreatment with the carboxylesterase inhibitor (TOTP) prior to RP73401 administration inhibited the hydrolysis of RP73401 to ADCP.

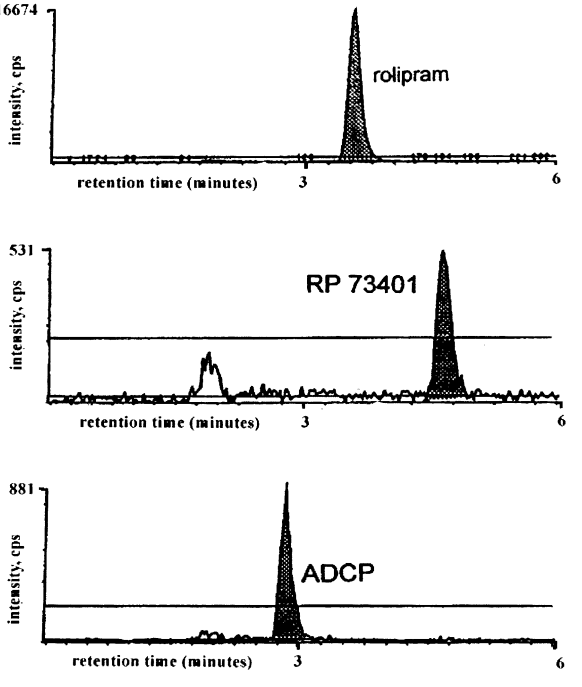

Fig. 4. Typical MRM chromatograms at the LLOQ.

Table 3
Rat and mouse plasma concentrations and AUC's

Dose (mg kg^{-1})	Species	Time (h)	Mean RP73401 (ng ml^{-1})	AUC RP73401 (h ng ml^{-1})	Mean ADCP (ng ml^{-1})	AUC ADCP (h ng ml^{-1})
250 MPK RP73401	Rat	0.5	32.82		12.02	
		1	26.5		16.99	
		2	42.28		24.68	
		4	77.5	12.9	46.78	74
0.1 MPK ADCP	Rat	0.5	na		66.24	
		1	na		68.55	
		2	na		42.42	
		4	na	na	24.3	143
1.0 MPK ADCP	Rat	0.5	na		506.6	
		1	na		436.54	
		2	na		398.87	
		4	na	na	237.9	127.1
250 MPK RP73401	Mouse	0.5	46.85		na	
		1	30.22		na	
		2	85.57		na	
		4	34.84	128	na	na
3.0 MPK ADCP	Mouse	0.5	na		110.37	
		1	na		201.05	
		2	na		195.38	
		4	na	na	106.21	652
10 MPK ADCP	Mouse	0.5	na		542.33	
		1	na		501.06	
		2	na		559.53	
		4	na	na	311.1	1394
2000 MPK RP73401	Mouse	0.5	209.52	na		
		1	258.15		na	
		2	252.9		na	
		4	299.11	814	na	na

The AUC's for rats administered ADCP (1 mg kg^{-1}) with and without TOTP pretreatment were comparable.

4. Conclusions

The analytical method described is suitable for the rapid and sensitive determination of RP73401 and ADCP in rat and mouse plasma. The concentration range which can be measured is 0.5–100 ng ml^{-1} for RP73401 and 5–500 ng ml^{-1} for ADCP. The method is rugged, linear, accurate, precise and shows acceptable recovery of the analytes and internal standard.

This LC/MS/MS method was used to analyze samples from two toxicokinetic studies. The first of these studies showed that although ADCP was present in the plasma of rats and mice administered a single, oral dose of RP73401, distinct differences in the relative amounts of ADCP were observed for the rat compared to the mouse. From these results, we conclude that the hydrolysis of RP73401 may be a species specific reaction.

Finally, this method has been used to generate data which indicate that pretreating rats with 300 mg kg^{-1} of TOTP (a carboxylesterase inhibitor) several hours prior to RP73401 dosing inhibits the hydrolysis of the amide linkage of RP73401.

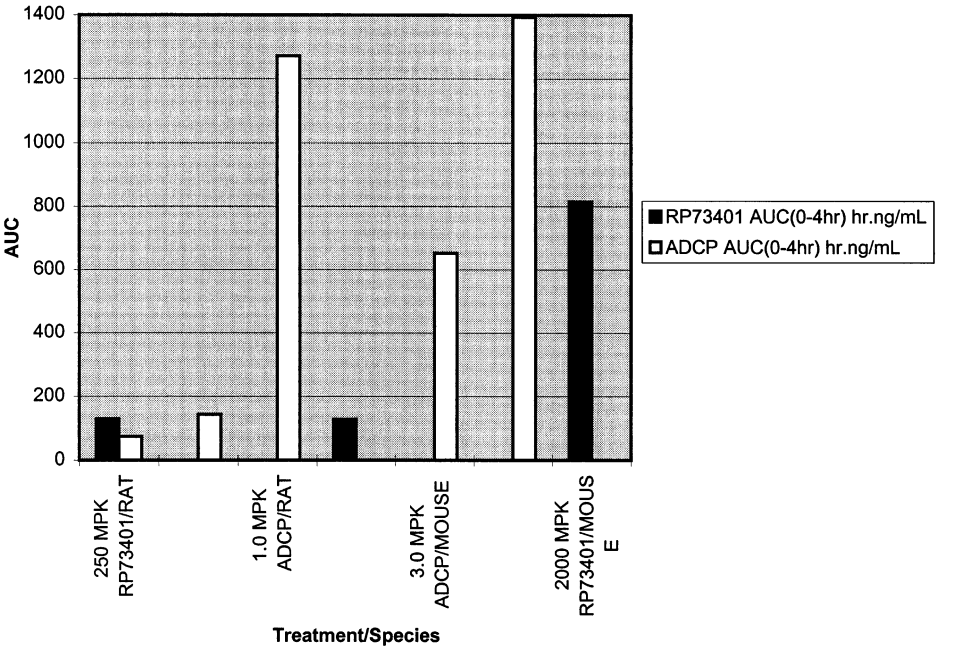

Fig. 5. RP73401 and ADCP AUC results from rat and mouse.

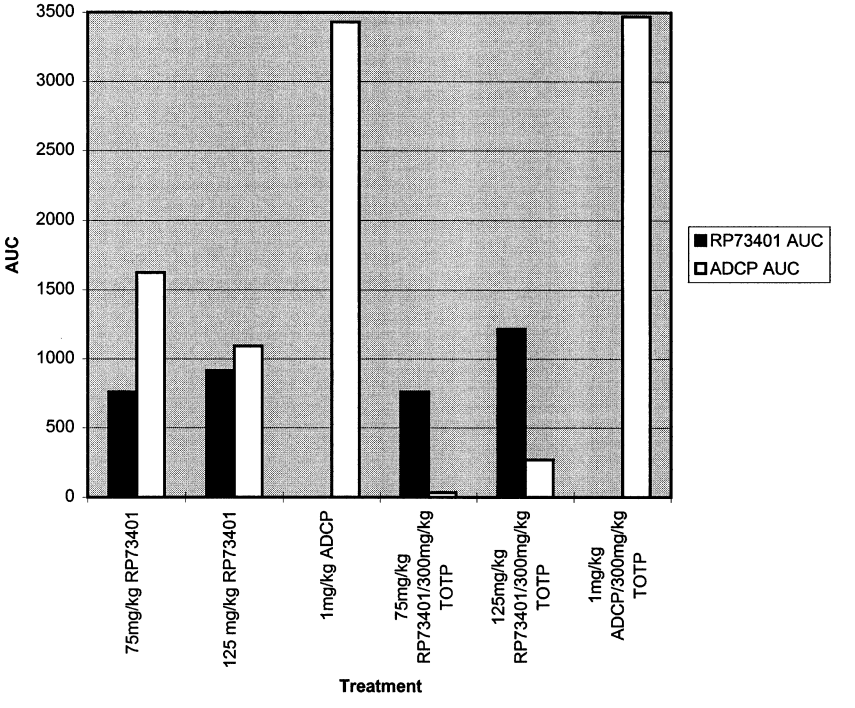

Fig. 6. Effect of TOTP pretreatment on RP73401 and ADCP AUC's in the rat.

From this data it is quite natural to conclude that the hydrolysis of RP73401 to ADCP may be mediated enzymatically by various carboxylesterases/amidases.

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