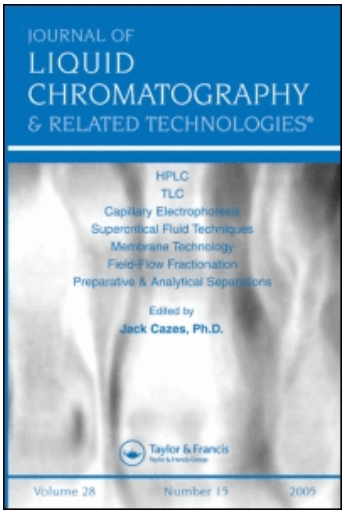

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PAIRED ION REVERSED-PHASE HPLC ASSAY FOR THE SIMULTANEOUS DETERMINATION OF IOTHALAMIC ACID AND PARA AMINOHIPPURIC ACID IN PLASMA

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ABSTRACT

An ion paired reversed - phase high performance liquid chromatographic assay for iothalamic acid and para aminohippuric acid in the same sample of plasma is described. The analysis uses one internal standard for both drugs. Sample preparation consists of precipitating plasma proteins with methanol and centrifuging to settle the proteins. The supernatant is evaporated and the residue reconstituted with mobile phase for injection. For HPLC a C_8 column and a mobile phase consisting of potassium phosphate buffer with dodecyl triethyl ammonium phosphate IP reagent, 22.5 % methanol and 2.5 % acetonitrile with UV detection at 254 nm was used. Coefficients of variation for the assay were in the range of 1.6 - 12.1% for iothalamic acid and 4.3 - 17.7% for para aminohippuric acid for four levels of concentration. Limits of quantitation were 3.0 μg/mL for iothalamic acid and 5.0 μg/mL for para aminohippuric acid. This isocratic HPLC assay is simple, rapid and relatively inexpensive.

INTRODUCTION

Iothalamic acid (Io, Figure 1A, 3-(acetylamino)-2,4,6,triiodo-5-[(methylamino) carbonyl]-benzoic acid) is a well known radiographic contrasting agent used in renal and urological procedures (1). It is also used as a marker, to replace inulin for measuring glomerular filtration rate (GFR). Para aminohippuric acid (PAH, Figure 1B, N-(4-aminobenzoyl)-glycine) is used to determine the effective renal plasma flow (ERPF) and as an ideal marker for renal blood flow (RBF) (2-4). The current methods used for these measurements are considered relatively invasive requiring IV infusion of markers and accurate urine collections. Measurements of creatinine clearance though widely used is prone to errors and can not detect minor changes in GFR (5).

Studies employing Io clearance for estimating GFR indicate, good correlation with inulin clearance and that this method is well tolerated by children and infants. Since the use of radiolabled iothalamic acid in patients is risky and subject to strict health and safety guidelines during usage, a method for measuring "cold" - Io as well as PAH in plasma and urine is advantageous.

Among the numerous methods reported for determining Io and PAH, those using radiolabled drugs are not suitable. Most other methods determine only one of these compounds (1,6-8). Many of the methods use colorimetry for PAH and several of the HPLC methods use immiscible organic solvent extractions to isolate the compounds from plasma and urine (1,6,8,9). While two methods used paired - ion mobile phases with reversed-phase columns for HPLC separation of either Io or PAH only one method reports the simultaneous assay for Io and PAH using reversed-phase HPLC (4,5,10). One significant problem with isocratic elution using the mobile phases reported in the earlier method, where the organic phase is below 10 % is that residual plasma and urine constituents are strongly retained on the column. Over time, this could cause column performance to deteriorate. Usually under these conditions a second pump

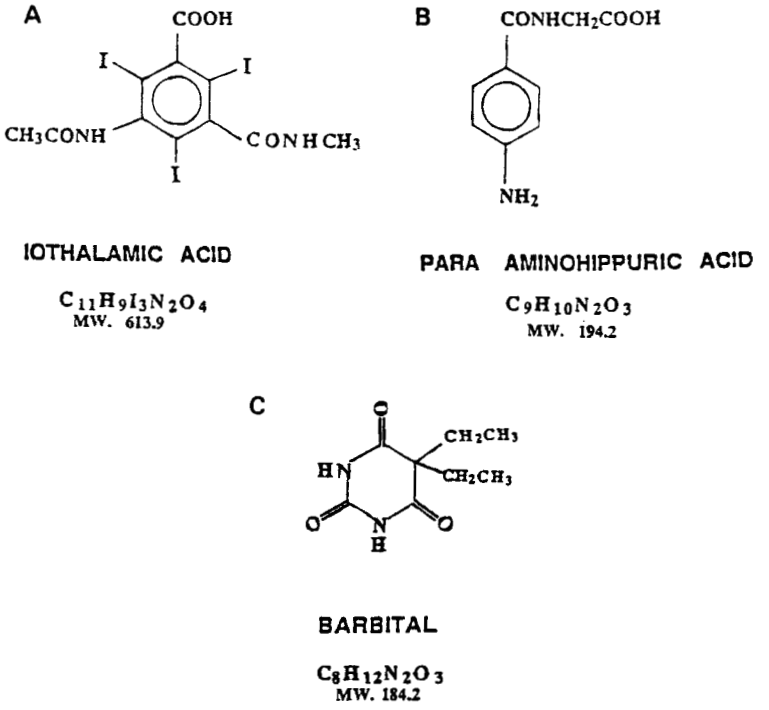

FIGURE 1. Chemical structures of (A) iothalamic acid, (B) p-aminohippuric acid and (C) 5,5 diethyl barbituric acid (barbital, internal standard).

coupled through a gradient controller is used to flush the column with a polar mobile phase for 2-5 minutes after each sample run followed by equilibration with the running mobile phase for at least 10 minutes. This technique requires either two pumps and a controller or a switching valve and a single pump coupled to a controller. A simple and less costly method was desirable to determine Io and PAH simultaneously. It was decided to increase the retention time of the compounds beyond 10 minutes and an ion-paired mobile phase was selected in order to achieve this. However p-aminobenzoic acid (PABA) was not adequately resolved from the two compounds, therefore barbital was used as the internal standard .

We decided to work in an isocratic mode with higher amounts of organic modifier in order to eliminate build up of a plasma residue on the column. An octyl C_8 reversed-phase column was selected in place of a C_{18} and the detector wavelength was fixed at 254 nm.

A complete assay validation, reported in this paper was performed. This was followed by determination of Io and PAH in a set of clinical samples .

EXPERIMENTAL

Apparatus

The HPLC system used consisted of a Waters M-510 HPLC pump, a model 712 WISP autoinjector, and a model 481 Lambda Max UV detector (Waters Assoc., Milford, MA). An HP 3396A-integrator (Hewlett Packard, Avondale,PA) was used for collecting the chromatographic data. The detector wavelength was 254 nm and the absorbance was set at 0.01 aufs. Separation was achieved with an Octyl (C-8) ,5µm particle size, 4.6 mm (i.d.) x 25cm reversed-phase column (Rainin Instrument Co. Inc., Woburn, MA).

Reagents

All solvents were of HPLC grade [Fischer Scientific Co., Fair Lawn, NJ]. Dodecyl triethylammonium phosphate [(Q-12, Ion pair reagent, 0.5M solution), Regis Chemical Co., Morton Grove, Il], potassium diacid phosphate, potassium mono acid phosphate [Fischer Scientific Co., Fair Lawn,NJ], iothalamic acid [Mallinckrodt Chemicals Inc., St.Louis, MO], p-aminohippuric acid sodium salt and barbital [Sigma Chemicals Co., St. Louis, MO], were used as received. Deionized distilled water was obtained from "Barnstead Nanopure" purification system (Barnstead Co., Boston, MA). Drug free human plasma was obtained from the Irwin Memorial Blood Center, San Francisco and Long Hospital Blood Bank, UC San Francisco, CA.

Mobile Phase

The mobile phase was composed of 22.5% methanol, 2.5% acetonitrile, and 1.75mM IP reagent in 10 mM potassium phosphate. It was prepared dissolving 6.44 g KH_2PO_4, 7.04 g K_2HPO_4 and 14 mL 0.5M Q-12 IP reagent solution in 4 L of deionized water and the pH was adjusted to 7.50. Three liters of the prepared buffer was filtered through a 0.22μm filter and mixed with 900 mL of methanol and 100 mL of acetonitrile. The mixture was degased by sonication under vacuum. The isocratic flow rate of the mobile phase was 1 mL/min.

Sample preparation

Calibration curve samples were prepared by spiking iothalamic acid, p-aminohippuric acid and the internal standard (barbital) into plasma. The plasma proteins in these samples, quality assuarance (QA) controls and clinical samples (100 μL each) were precipitated out with 500 μL aliquots of HPLC grade methanol. Samples were vortexed for 15 seconds and centrifuged for 10 minutes at 2500 rpm. The supernatants were concentrated under nitrogen and residues were reconstituted in running buffer. Fifteen to twenty micro liter samples were injected onto the column for analysis.

All clinical samples, QA samples and stock solutions of compounds were stored at -20°C until analysis. Spiked samples for calibration curves and controls at four different drug concentrations were prepared with drug free plasma and were spiked with separately prepared drug solutions.

RESULTS

Figure 2a and 2b represent typical chromatograms of the internal standard in blank plasma and the two compounds and internal standard in plasma, respectively. The mean retention times of p-aminohippuric acid, barbital and iothalamic acid are 12.0, 15.9 and 18.2 minutes

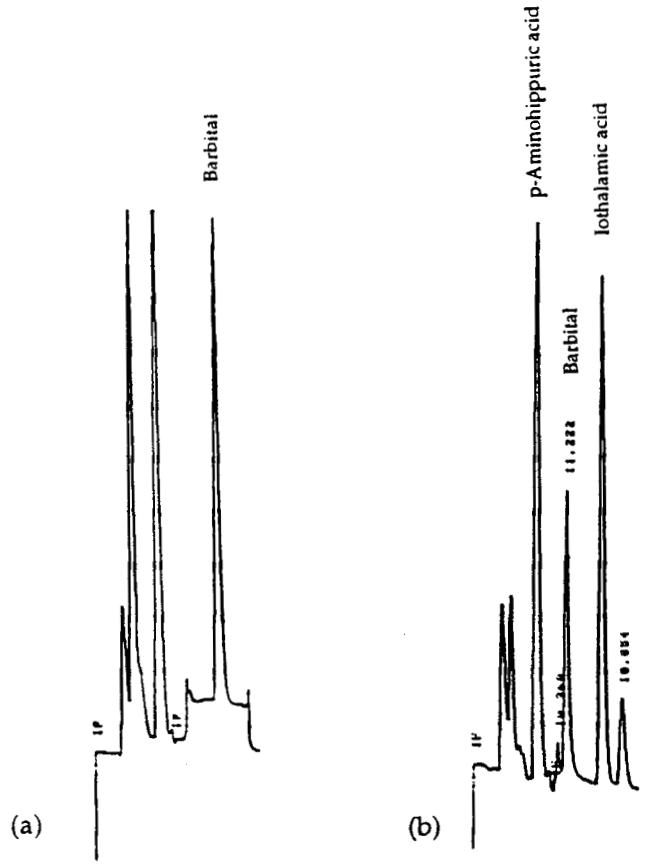

FIGURE 2. (a). Chromatogram of blank plasma with internal standard.

(b). Chromatogram of iothalamic acid, p-aminohippuric acid and the internal standard in plasma.

respectively. (However, fluctuations of these retention times are observed due to the variation of temperature and column performance). The concentration data used for the calibration curves are presented in Table 1. (5.0 to 200.0 μg/mL for Io and 10.0 to 380.0 μg/mL for PAH). Even though the limits of quantitation were 3.0 μg/mL (CV% 10.03) and 5.0 μg/mL (CV% 9.56) for these two drugs, these lowest points were omitted from the calibration curves.

TABLE 1

Data Used for Calibration Curves of Iothalamic Acid and p-Aminohippuric Acid in Plasma.

Iothalamic Acid			p-Aminohippuric Acid		
Conc. Io. µg/mL	Peak Height Ratic (Io/Barb.)	Calc. Conc. Io,µg/mL	Conc. PAH µg/mL	Peak Height Ratio (PAH/Barb)	Calc. Conc. PAH,µg/mL
0.00	0.00	0.00	0.00	0.00	0.00
5.00	0.16	4.72	10.00	0.41	9.27
10.00	0.29	9.75	25.00	0.98	23.23
20.00	0.55	20.10	50.00	2.04	49.45
40.00	1.03	39.39	100.00	4.20	102.30
80.00	2.06	80.28	200.00	8.19	200.48
140.00	3.62	142.54	300.00	12.43	304.41
200.00	5.01	198.26	380.00	15.34	375.87

Two typical calibration graphs used for the calculation of concentration of the two compounds are presented in Figures 3 and 4. Linear regression of peak height ratio vs. compound - concentration gives typical coefficients of determination (r^2) of 0.999 or better for iothalamic acid and p-aminohippuric acid.

Variability Studies

Interday and intraday variability was studied using frozen controls at four concentratons, extra-low, low, medium and high. Five samples from each concentration were assayed for both interday and intraday studies. For interday variability, six calibration curves on six different days were used and one calibration curve was used for the intraday variability study. The ranges for the coefficients of variation were 6.33 to 12.10% for iothalamic acid and 4.30 to 17.72% for p-aminohippuric acid for the interday studies as shown in Tables 2 and 3, respectively. Coefficients of variation ranged from 1.60 to 5.00 % for iothalamic acid and 7.00 to 8.41 % for p-aminohippuric acid for the intraday study as shown in Tables 4 and 5, respectively.

Recovery

Assay recovery was measured by comparing the peak height ratios of the compounds to barbital, at four different compound-concentrations in plasma and in aqueous compound solutions spiked at the same concentration. The internal standard was added to the plasma samples only after the supernatant was decanted from the precipitated proteins. Both sets of samples were then evaporated and reconstituted in running buffer for injection. The mean % recovery was calculated as follows:

$$\% \text{ Recovery} = \frac{\text{peak height ratio of compound in plasma}}{\text{peak height ratio of compound in water}} \times 100$$

The mean recovery was 91.1 % for iothalamic acid and 79.2 % for p-aminohippuric acid.(Tables 6 and 7)

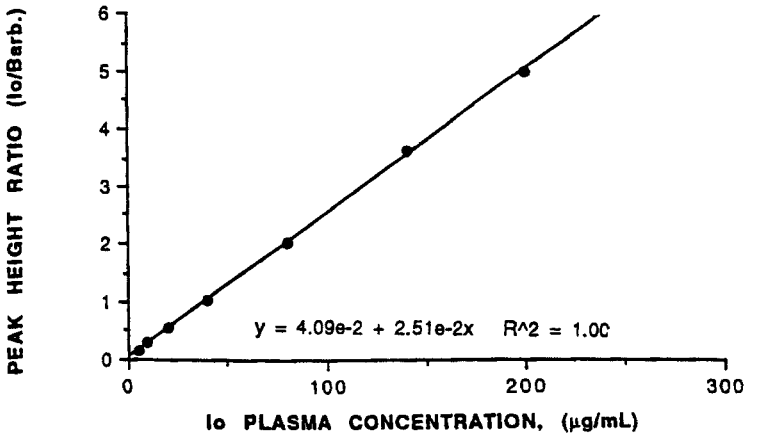

FIGURE 3. Calibration curve of iothalamic acid in plasma.

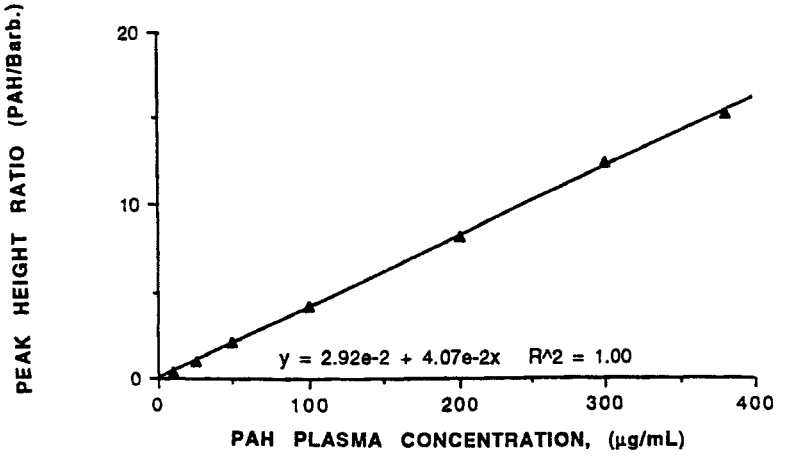

FIGURE 4. Calibration curve of p-aminohippuric acid in plasma.

TABLE 2
Interday Variation of Iothalamic Acid in Plasma

	CONCENTRATION* (μg/mL)			
	EXTRA-LOW [12.0]	LOW [35.0]	MEDIUM [85.0]	HIGH [150.0]
1	12.92	38.03	81.84	154.92
2	13.21	37.52	82.17	148.17
3	12.48	37.41	82.23	150.23
4	12.44	37.22	81.08	149.15
5	13.13	37.22	79.43	145.89
Mean	12.84	37.50	81.35	149.67
SD	1.55	2.37	5.79	14.46
%CV	12.10	6.33	7.14	9.63

*Each value represents mean of n=5

TABLE 3
Interday Variation of p-Aminohippuric Acid in Plasma

	CONCENTRATION* (μg/mL)			
	EXTRA-LOW [25.0]	LOW [70.0]	MEDIUM [180.0]	HIGH [320.0]
1	25.12	73.74	186.13	333.58
2	23.87	75.43	188.87	336.69
3	23.88	73.47	186.57	339.78
4	25.21	73.86	179.83	340.79
5	24.76	74.56	190.68	339.01
Mean	24.57	74.21	186.41	337.97
SD	4.34	3.19	22.32	34.12
%CV	17.72	4.30	11.96	10.11

*Each value represents mean of n=5

TABLE 4
Intraday Variation of Iothalamic Acid in Plasma

	CONCENTRATION* (μg/mL)			
	EXTRA-LOW [12.0]	LOW [35.0]	MEDIUM [85.0]	HIGH [150]
1	14.03	37.30	78.49	141.50
2	14.12	37.05	78.33	132.15
3	14.12	35.65	72.29	135.88
4	13.77	36.29	75.72	138.17
5	12.85	36.53	80.11	142.98
6	13.47	36.39	75.16	124.51
Mean	13.73	36.54	76.68	135.87
SD	0.50	0.59	2.84	6.79
%CV	3.62	1.60	3.70	5.00

*Each value represents mean of n=5

TABLE 5
Intraday Variation of p-Aminohippuric Acid in Plasma

	CONCENTRATION (μg/mL) (n=5)			
	EXTRA-LOW [25.0]	LOW [70.0]	MEDIUM [180.0]	HIGH [320.0]
1	21.72	65.85	180.88	298.95
2	20.09	67.48	172.34	282.84
3	17.90	55.77	176.21	281.33
4	20.08	63.95	165.54	280.74
5	17.22	58.32	155.18	295.06
6	19.63	61.85	163.43	298.97
Mean	19.44	62.20	163.93	289.65
SD	1.64	4.49	12.26	19.68
%CV	8.41	7.22	7.48	7.00

TABLE 6
Recovery of Iothalamic Acid from Plasma

Conc. Io	Peak Height Ratio (Plasma)		Peak Height Ratio (Water)		Recovery %
µg/mL	Mean*	Std.Dev.	Mean*	Std. Dev.	
12	0.34	0.0004	0.36	0.0239	96.6
35	0.81	0.0098	0.95	0.0060	85.7
85	1.92	0.0421	2.09	0.0782	92.0
150	3.97	0.0980	4.40	0.0373	90.1
Overall recovery of Iothalamic Acid					**91.1.%**

* n=5

TABLE 7
Recovery of p-Aminohippuric Acid from Plasma

Conc. PAH	Peak Height Ratio (Plasma)		Peak Height Ratio (Water)		Recovery %
µg/mL	Mean*	Std. Dev.	Mean*	Std. Dev.	
25	0.86	0.0021	1.20	0.0230	71.39
70	2.42	0.0225	3.11	0.0321	77.77
180	6.30	0.1040	7.50	0.2546	84.05
320	11.53	0.3022	14.48	0.0785	79.60
Overall recovery of p-aminohippuric acid					**78.2.%**

* n=5

Table 8
Storage Stability (24 hours at RT) of Iothalamic Acid and p-Aminohippuric Acid in Plasma

Conc.,µg/mL	Percent Change Io*	Conc.,µg/mL	Percent Change PAH*
12.0	13.78	25.0	-19.27
35.0	10.09	70.0	1.63
85.0	9.12	180.0	4.53
150.0	1.67	320.0	0.37

*Each value represents mean of n=5

Table 9
Heat Deactivation Stability of Iothalamic Acid and p-Aminohippuric Acid in Plasma

Conc.,µg/mL	Percent Change Io*	Conc.,µg/mL	Percent Change PAH*
12.0	4.92	25.0	4.16
35.0	1.48	70.0	-12.02
85.0	-2.85	180.0	-15.70
150.0	-3.74	320.0	-4.10

*Each value represents mean of n=5

Stability

The stability of the two compounds in plasma during storage at room temperature for 24 hours and during heat deactivation at 56-58°C for 55 minutes were evaluated (Tables 8 and 9 respectively). Five samples each from the four controls were used for both stability studies. Further, we investigated the stability of the two compounds during three freezing and thawing cycles. Our results showed that there was a loss of both compounds to the extent of 3% for Io and 10.2 % for PAH after the third cycle (Table 10).

Table 10

Freeze - Thaw Stability of Iothalamic Acid and p-Aminohippuric Acid in Plasma

Percent Change Io Conc.				Percent Change PAH Conc.			
	Freeze-Thaw Cycle				Freeze-Thaw Cycle		
Conc.,µg/mL	1	2	3	Conc.,µg/mL	1	2	3
12.0	0.00	24.84	6.75	25.0	0.00	28.49	-9.77
35.0	0.00	-1.94	-5.02	70.0	0.00	-3.92	-4.52
85.0	0.00	-2.03	-5.46	180.0	0.00	-2.73	-3.83
150.0	0.00	-7.50	-8.46	320.0	0.00	-6.96	-22.92
Mean	**0.00**	**3.34**	**-3.05**		**0.00**	**3.72**	**-10.26**

DISCUSSION

The forgoing method is the first reported, for determining iothalamic acid and p-aminohippuric acid in the same plasma sample using an isocratic paired ion reverse phase HPLC assay. The use of barbital to replace PABA as the internal standard has the advantage of eliminating interferences by metabolites of these drugs, including PABA. The recently marketed ion pairing reagent dodecyl trimethylammonium phosphate is needed only in small quantities. The long 12- carbon chain, bonds more effectively to the C-18 group on the column packing thus giving better retention properties for acidic drugs. The main advantage of the method is that gradient - elution or dual pump operations are eliminated by having a polar mobile phase containing 20-25% methanol. This results in improved column performance over long periods of time without frequent washing of the column to remove interfering substances usually present in bio-matrices.

The assay method has a lower limit of quantitation of 3.0μg/mL for iothalamic acid and 5.0μg/mL for p-aminohippuric acid. The recovery of the compounds during extraction were very satisfactory and their stability in plasma was within acceptable limits.

Using this method, plasma and urine samples from twenty-four subjects with normal and impaired renal function were analysed for Io and PAH. During the course of a pharmacokinetic study the renal clearances of iothalamic acid and p-aminohippuric acid were determined (11).

The validation of this assay was carried out according to the guidelines put forward by the conference on Analytical Methods Validation; Bioavailability, Bioequivalence and Pharmacokinetic Studies sponsored by FDA, AAPS and AOAC among others [12]. It is noteworthy that, this reversed -phase ion pair HPLC method with isocratic elution is a simple, rapid, relatively inexpensive and accurate method for simultaneous determination of these two compounds in human plasma.

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REFERENCES

1. F.Gaspari, L.Mainardi, P.Ruggenenti and G.Remuzzi, J.Chromatogr., 570(2), (1991), 435.

2. H.Chassis, H.A.Ranges, W.Goldrig and H.W.Smith, J.Clin.Invest., 17, (1938), 683.

3. H.W.Smith, N.Finkelstein, L.Aliminosa, B.Cranford and M.Graber J.Clin.Invest., 24, (1946), 388.

4. P.D.Jenny, A.Weber and A.L.Smith, J.Chromatogr., 490,(1989), 213.

5. T.Prueksaritanont, M.Chen and W.L.Chiou, J.Chromatogr., 306, (1984), 89.

6. R.N.Dalton, M.J.Wiseman, C.Turner and G.Viberti, Kidney Int., 34, (1988), 117.

7. L.B.Schwartz, B.L.Gewertz and M.G.Bissell, Clin.Chem., 34/1, (1988) 165.

8. M.M.Reidenberg, B.J.Lorenzo, D.E.Drayer, J.Kluger, T.Nestor, J.C.Regnier, B.A.Kowal and I.Bekersky,Ther.Drug Monit., 10, (1988), 434.

9. M.Kiguchi and J.Sudo , J.Toxicol.Sci., 12, (1987),30

10. S.Boschi and B.Marchesini, J.Chromatogr., 224, (1981) ,139.

11. Drug Study (1992) #DSU 87-45/PK6 of the Drug Studies Unit, School of Pharmacy, University of California, San Francisco, CA 94143.

[12].V.P.Shah, K.K. Midha, S. Dighe, I.J.McGilveray, J.P.Skelly, A.Yakobi, T.Layloff, C.T.Wiswanathan, C.Edgar Cook, R,D.McDowall, K.A.Pittman, and S. Spector. *J.Pharm. Sci.*, 81-3, 309, (1992).

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