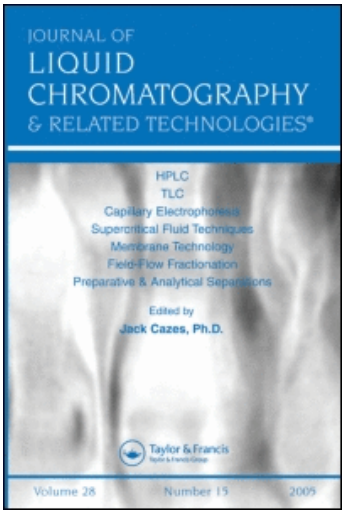

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ABSTRACT

An isocratic high performance liquid chromatographic (HPLC) method for the quantification of cyproheptadine in human milk and plasma respectively using diphenylpyraline as an internal standard is described. N-Pentane containing 2% v/v isopropyl alcohol was used as an extraction solvent to prevent adsorption of the compounds onto glass. To compare the extraction efficiency between the solvent and solid phase techniques, 3 mL sized C_{18} Supelco cartridges were used in processing samples by SPE method.

A reversed phase octyl-ODP column (150X4.6 mm ID) was used with a mobile phase consisting of acetate buffer of 0.05 M - methanol (68:32 v/v), pH 3.6. Good quantitation was obtained in the concentration range 50-739 ng/mL by the use of UV detection at 254 nm with the sensitivity set at 0.002 AUFS.

The overall recovery was found to be 86.76% in milk, 91.54% in plasma by solvent extraction, and 83.74% in milk, 89.77% in plasma by SPE respectively, with coefficients of variation <1.53%. The limit of detection was estimated as 15 ng/mL.

INTRODUCTION

Cyproheptadine, 1-Methyl-4-(5H-dibenzo[a,d] cycloheptenylidine) piperidine, is a potent antagonist of histamine and serotonin[1] and is widely used as an appetite stimulant.[2] It has been used as an antipruritic agent[3] and is reported to be useful in treating post-gastrectomy dumping syndrome.[4]

Several analytical methods have been reported for the separation of cyproheptadine by gas chromatography,[5,6] gas-liquid chromatography[7] and high performance liquid chromatography.[8-10] The HPLC methods require a tedious multi step liquid-liquid extraction procedure.[8,9] Also, a solid phase extraction procedure in urine is described.[10] In the present report, we are presenting a simple and rapid liquid-liquid extraction method in connection with an easy, effective, and convenient solid phase extraction technique for quantitating cyproheptadine in human milk and plasma, respectively, at ppb concentrations. The chromatographic system is a modification of a previously described chromatographic system[10] by using the internal standard quantification method.

EXPERIMENTAL

Apparatus

The apparatus used for HPLC consisted of a Shimadzu series LC-6A with two pressure pumps and a UV spectrophotometric detector module SPD-6AV was isocratically operated. A reversed phase octyl-ODP column Asahipak ODP.50, Asahi, Japan (150X4.6 mm ID) with thermostated oven at 40°C, model CTO-6A and an injection Rheodyne valve with a 20 μL loop was used. The chromatograms were recorded on a chart paper, module Chromatopac C-R 6A at a speed of 1 cm/min. All were products of Shimadzu instruments corporation (Kyoto-Japan).

Chemicals, Solutions, and Samples:

Cyproheptadine hydrochloride and diphenylpyraline hydrochloride were obtained from Sigma (St. Louis, MO, USA) and used without further

purification. HPLC grade methanol and water were used throughout. All other chemicals were of analytical reagent grade and were used as received. Human milk and plasma were supplied by a maternity hospital.

Stock solutions were prepared by accurately weighing the appropriate amounts of cyproheptadine and diphenylpyraline and dissolving each separately in methanol in a 50 mL volumetric flask to prepare 1 mg/mL stock standard solutions. Then an intermediate solution was prepared and working standard solutions of cyproheptadine were prepared from the intermediate solution by sequential dilutions with methanol to give concentrations of 53, 106, 211, 317, 422, 528, 634, 739 ng/mL, each containing 400 ng/mL of diphenylpyraline that was used as the internal standard. The absolute recoveries of cyproheptadine and the internal standard from human milk and plasma samples were similar and ranged from 78-95%.

Chromatographic Conditions

The mobile phase was 0.05 M acetate buffer-methanol (68:32). The apparent pH was adjusted to 3.6 using glacial acetic acid after mixing appropriate volumes of aqueous ammonium acetate and methanol. The column was equilibrated with the eluting solvent by pumping the mobile phase at a rate of 0.3 mL/min and degassed by slowly bubbling helium gas overnight.

The flow rate was set at 1.4 mL/min during analysis and detection was performed at 254 nm with a sensitivity of 0.002 AUFS. The wavelength was chosen at 254 nm as internal standard diphenylpyraline exhibits at this wavelength its maximum absorption, whereas, absorption of cyproheptadine in the same wavelength is reasonably high.

Extraction Procedure

Liquid-liquid extraction method (LLE)

1.0 mL volume of the eight working standard solutions of cyproheptadine, of concentrations ranging from 53 to 739 ng/mL containing an amount of 400 ng of diphenylpyraline were added into 1 mL of human milk or plasma samples respectively in a 12 mL glass centrifuge tube. 0.5 mL of 3 M NaOH solution was added to the solution and mixed briefly to make it alkaline. Then 3 mL of n-pentane containing 2% v/v of isopropyl alcohol was also added, and the mixture was vortexed mixed at high speed for 2 mins and centrifuged at 2000 rpm for 5 min in order to separate the two phases clearly.

The upper organic layer was transferred into another tube and evaporated to dryness at ambient temperature under stream of nitrogen for almost ten minutes. The residue was dissolved by the addition of 1 mL of methanol and an aliquot of 20 μL was injected onto the HPLC column for analysis.

Solid phase extraction procedure (SPE)

1.0 mL volume of cyproheptadine working standard solutions of concentrations ranging from 53 to 739 ng/mL and containing a fixed amount of 400 ng of diphenylpyraline was evaporated to dryness, each in a 12 mL glass centrifuge tube under nitrogen. Then 1.0 mL of human milk or plasma samples respectively and 3 mL of acetonitrile were added into the tubes, vortexed briefly and centrifuged at 2000 rpm for 5 mins to precipitate proteins. The upper liquid layer was removed into a clean 12 mL centrifuge tube and was combined with water (1:2), mixed thoroughly and treated by solid phase extraction using 3 mL C_{18} Supelco cartridges.

The sample was slowly passed through pre-conditioned cartridges and washed with 2 mL of water before the final elution of the compounds with 6 mL of methanol. The methanolic solution was evaporated to dryness under nitrogen at 45°C. The residue was redissolved in 1 mL of methanol and aliquots of 20 μL were injected onto the analytical column.

RESULTS AND DISCUSSION

The use of an octyl-ODP column in a reversed phase mode and by isocratic elution with a mobile phase of 32:68 methanol-acetate buffer 0.05 M, pH 3.6, produced good separations of both compounds. Typical chromatograms of cyproheptadine and diphenylpyraline extracts from plasma is shown in Figure 1. The retention time was approximately 13.1 mins for cyproheptadine and 7.96 mins for diphenylpyraline, respectively. Their resolution factor was 2.38.

The choice of extraction conditions is based on compromise between extraction yield and selectivity of extraction. In the liquid-liquid extraction method, the use of n-pentane in alkaline milk and plasma afforded a cleaner and higher yield of the extracts than several other solvents from our preliminary studies. The addition of 2% v/v isopropyl alcohol to the n-pentane, minimized the adsorption of the compounds onto the glass surface, where as in the solid phase extraction procedure, the precipitation of proteins from plasma and milk samples prior to extraction helped to avoid the blocking of the cartridges by protein materials.

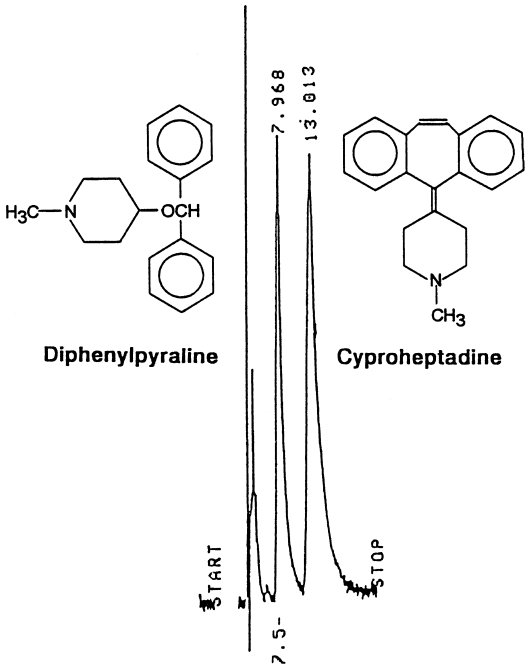

Figure 1. Representative chromatogram of plasma extracts spiked with cyproheptadine and diphenylpyraline as internal standard.

Moreover, the addition of water after removing the protein matrix was necessary to hold back the compounds of interest on the cartridge material: Because early studies have shown that acetonitrile has the ability to elute cyproheptadine and its internal standard together with undesired biological material from the extraction tubes.

A linear relationship (r=0.9995) was found over the concentration range 53-739 ng/mL after extraction from both milk and plasma samples. The recovery and accuracy of the assay was assessed by using a cyproheptadine calibration graph of the peak height ratio of cyproheptadine to the internal standard after direct injection of methanolic solutions containing known quantities of cyproheptadine versus concentration. Using the characteristics of this calibration graph, the amount of the drug recovered after extracting spiked plasma and milk samples containing equivalent amounts of the drug was calculated from the standard calibration graph. At each of the eight cyproheptadine concentrations used, four replicate samples were measured. Results of the standard calibration and recovery data are given in Tables 1 to 4.

Table 1

Standard Calibration and Recovery Data for Cyproheptadine in Spiked Human Milk Treated by Liquid-Liquid Extraction*

Added (ng/mL)	Mean Peak Height Ratio	Found (Mean±SD) (ng/mL)	CV (%)	Recovery (%)
53	0.269	44.53±0.42	0.94	84.02
106	0.538	90.54±1.06	1.77	85.42
211	1.077	182.80±1.78	0.97	86.61
317	1.641	279.20±2.85	1.02	88.09
422	2.179	371.30±3.68	0.99	87.98
528	2.718	463.50±4.22	0.91	87.78
634	3.205	546.80±5.69	1.04	86.24
739	3.743	638.80±7.08	1.11	86.44

* (n=4); $y= 50.72\ 10^{-3}x + 12.95\ 10^{-3}$ (r=0.9998); Average Recovery = 86.57%; Average C.V. = 1.02%.

Table 2

Standard Calibration and Recovery Data for Cyproheptadine in Spiked Plasma Treated by Liquid-Liquid Extraction*

Added (ng/mL)	Mean Peak Height Ratio	Found (Mean±SD) (ng/mL)	CV (%)	Recovery (%)
53	0.300	49.83±0.91	1.83	94.02
106	0.590	99.39±1.66	1.67	93.76
211	1.192	202.40±2.18	1.08	95.93
317	1.750	297.90±4.36	1.46	93.97
422	2.312	394.00±4.89	1.24	93.37
528	2.910	496.30±8.78	1.77	94.00
634	3.507	598.40±9.45	1.58	94.39
739	3.900	665.70±10.9	1.64	90.08

* (n=4); $y=5.351\ 10^{-3}\ x + 44.27\ 10^{-3}$ (r=0.9993); Average Recovery=93.69%; Average C.V.=1.53%.

Table 3

Standard Calibration and Recovery Data for Cyproheptadine in Spiked Human Milk Treated by Solid Phase Extraction*

Added (ng/mL)	Mean Peak Height Ratio	Found (Mean±SD) (ng/mL)	CV (%)	Recovery (%)
53	0.250	41.28±0.29	0.70	77.87
106	0.525	88.32±0.50	0.57	83.31
211	1.025	173.90±1.63	0.94	82.39
317	1.500	255.10±3.00	1.18	80.48
422	2.050	349.20±3.52	1.00	82.75
528	2.600	443.30±3.97	0.90	83.95
634	3.100	528.80±4.48	0.85	83.41
739	3.600	614.40±6.33	1.03	83.13

* (n=4); $y=4.895\ 10^{-3}x + 10.65\ 10^{-3}$ (r=0.9998); Average Recovery=82.16%; Average C.V.=0.90%.

Table 4

Standard Calibration and Recovery Data for Cyproheptadine in Spiked Plasma Treated by Solid Phase Extraction*

Added (ng/mL)	Mean Peak Height Ratio	Found (Mean±SD) (ng/mL)	CV (%)	Recovery (%)
53	0.275	45.55±0.48	1.05	87.94
106	0.557	93.79±0.96	1.02	88.48
211	1.075	182.40±1.85	1.01	86.45
317	1.711	291.20±3.90	1.06	91.86
422	2.248	383.10±3.70	0.97	90.78
528	2.808	478.90±5.28	1.10	90.63
634	3.378	576.40±5.99	1.03	90.91
739	3.813	650.80±7.41	1.14	88.06

* (n=4); $y=5.248\ 10^{-3}x + 8.6\ 10^{-3}$ (r=0.9995); Average Recovery=89.14%; Average C.V.=1.05%.

The overall recovery of the drug was calculated by plotting a standard calibration of the «added» versus the «found» concentrations of cyproheptadine in plasma and milk samples respectively by both liquid-liquid and solid phase extraction procedures. These gave linear relationships y=0.8676x+0.737 (r=0.9998) and y=0.9154x+6.061 (r=0.9993) for milk and plasma samples by liquid-liquid extraction method and y=0.8374x - 3.307 (r=0.9998) and y=0.8977x - 0.020 (r=0.9995) for milk and plasma samples respectively, treated by solid phase extraction technique.

Therefore, the slopes (0.8676 for milk and 0.9154 for plasma by LLE and 0.8374 for milk and 0.8977 for plasma by SPE) of these regression lines were used as estimates of the overall recovery for cycloheptadine 86.76% in milk, 91.54% in plasma by LLE and 83.74% in milk and 89.77% in plasma by SPE respectively.[10]

The accuracy and precision of the method were determined by spiking plasma and urine samples with cyproheptadine standards at two concentration levels (106 and 422 ng/mL), each containing 400 ng/mL of diphenylpyraline as internal standard.

Extraction and HPLC determinations were carried out as described above. The within-day accuracy and precision for each concentration level were obtained from four analyses of the samples of the same concentration treated by each method of extraction. The overall accuracy and precision for each concentration level were obtained from twelve analyses of samples of the same concentration on three different days (4x3) within 1 month. Therefore, for the assessment of the mean precision within 1 month at two concentration levels, 48 plasma and milk samples each were treated by the two methods of extraction. The results are presented on Tables 5 to 8. The detection limit of the assay defined as the minimum drug concentration to produce twice the signal to noise ratio at 0.002 AUFS, was found to be 15 ng/mL for both plasma and milk samples.

The liquid-liquid and solid phase extraction methods described in this report gave high yields of cyproheptadine and diphenylpyraline extracts from human milk and plasma samples respectively. Both methods of extraction were simple, convenient, time and cost saving, utilizing less expensive solvents.

Although several authors[11-14] had favoured SPE over LLE, we obtained higher recoveries of cyproheptadine and diphenylpyraline with liquid-liquid extraction compared with solid phase extraction in both human milk and plasma samples. However, unlike the liquid-liquid extraction method which gave variable results, SPE gave more reproducible results.

Table 5

Accuracy and Precision for the Determination of Cyproheptadine in Spiked Human Milk Treated by Liquid-Liquid Extraction*

Added (ng/mL)	Day	Accuracy Mean Found Conc. (ng/mL)*	Precision SD	Precision CV%	Mean Conc. Found ±SD (ng/mL)**
106	1	90.54	1.37	1.51	
	2	91.22	1.41	1.55	90.97±1.44
	3	91.14	1.56	1.71	
422	1	372.27	4.13	1.11	
	2	370.85	4.47	1.21	371.26±4.16
	3	370.66	3.89	1.05	

* n = 4.
** n = 12.

Table 6

Accuracy and Precision for the Determination of Cyproheptadine in Spiked Plasma Treated by Liquid-Liquid Extraction

Added (ng/mL)	Day	Accuracy Mean Found Conc. (ng/mL)*	Precision SD	Precision CV%	Mean Conc. Found ±SD (ng/mL)**
106	1	97.75	1.76	1.80	
	2	99.20	1.65	1.66	90.00±1.60
	3	100.06	1.38	1.38	
422	1	395.15	4.80	1.22	
	2	395.44	4.69	1.22	395.56±4.91
	3	396.09	5.24	1.32	

* n =4.
** n=12.

Table 7

Accuracy and Precision for the Determination of Cyproheptadine in Spiked Human Milk Treated by Solid Phase Extraction

Added (ng/mL)	Day	Accuracy Mean Found Conc. (ng/mL)*	Precision SD	Precision CV%	Mean Conc. Found ±SD (ng/mL)**
106	1	88.52	1.02	1.15	
	2	90.18	1.18	1.31	89.48±1.16
	3	89.73	1.25	1.39	
422	1	350.43	3.76	1.07	
	2	352.16	3.61	1.03	350.51±3.90
	3	348.95	4.34	1.26	

* n = 4.
** n = 12.

Table 8

Accuracy and Precision for the Determination of Cyproheptadine in Spiked Plasma Treated by Solid Phase Extraction

Added (ng/mL)	Day	Accuracy Mean Found Conc. (ng/mL)*	Precision SD	Precision CV%	Mean Conc. Found ±SD (ng/mL)**
106	1	95.45	1.04	1.09	
	2	93.80	1.18	1.41	94.41±1.18
	3	93.98	1.17	1.25	
422	1	380.78	4.19	1.10	
	2	382.67	3.96	1.03	381.65±4.34
	3	381.50	4.87	1.28	

* n = 4.
** n = 12.

CONCLUSION

The major advantages of both liquid-liquid and solid phase extraction techniques described are their simplicity, rapidity, and cost savings. The proposed method provides good sensitivity, reproducibility, and repeatability for the HPLC analysis of cyproheptadine in human milk and plasma samples using diphenylpyraline as the internal standard.

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