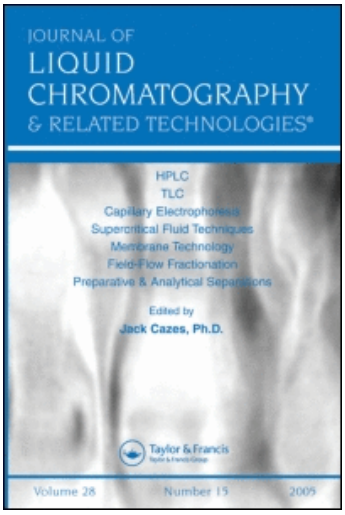

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Flow Injection Analysis of Carboxylic Acid Drugs Using Cationic Dyes and On-Line Ion-Pair Extraction

James T. Stewart[a]; James R. Lang[a]; Irwin L. Honigberg[a]
[a] Department of Medicinal Chemistry, College of Pharmacy The University of Georgia, Athens, Georgia

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FLOW INJECTION ANALYSIS OF CARBOXYLIC ACID DRUGS USING CATIONIC DYES AND ON-LINE ION-PAIR EXTRACTION

JAMES T. STEWART, JAMES R. LANG AND IRWIN L. HONIGBERG
Department of Medicinal Chemistry
College of Pharmacy
The University of Georgia
Athens, Georgia 30602

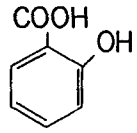
ABSTRACT

A flow injection system containing an on-line ion-pair extractor has been designed for the analysis of carboxylic acid drugs using either spectrophotometric detection with gentian violet as counterion or fluorescence detection with acridine orange. Salicylic acid, valproic acid, and ibuprofen were selected as model drugs. A mobile phase of 90:10 aqueous pH 7 phosphate buffer-absolute methanol was pumped through the system at 1 ml/min. A chloroform solution of the cationic dye was pumped into the mobile phase at 1.25 ml/min and the chloroform layer containing the dye-drug ion-pair separated prior to detection. Peak height and absorbance were linear for salicylic acid, valproic acid, and ibuprofen in the 0.5-10, 5-50, and 1-10 µg/ml ranges, respectively. Peak height and fluorescence were linear for salicylic acid, valproic acid, and ibuprofen in the 0.13-5, 2.5-50, and 0.5-20 µg/ml ranges, respectively. Accuracy and precision for the spectrophotometric assays were in the 2-6% and 3.3-6.6% ranges, respectively, and in the 0.3-4% and 1.3-5.4% ranges, respectively, for the fluorescence assays. Peak height and absorbance were also shown to be linear in the

16-500 µg/ml range for prostaglandin $PGF_{2\alpha}$ with accuracy and precision of 1-3% and 5-6%, respectively, for spiked samples. Commercial dosage forms containing valproic acid and ibuprofen were assayed by the spectrophotometric assay and found to be within acceptable USP limits. Spiked ibuprofen samples at the 5 and 10 µg/ml level were assayed using an octadecylsilane column inserted into the flow injection system. One to two percent accuracy and 2.5-5% precision were obtained for the drug using the FIA-column system.

INTRODUCTION

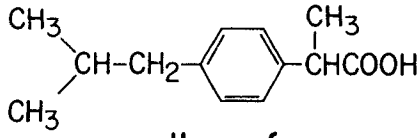
The popularity of ion-pairing as an analytical technique has increased significantly over the past 10-15 years. Much of the theory and application of ion-pair techniques has been attributed to Schill and coworkers in Sweden (1-4). One distinct advantage of using ion-pairing in pharmaceutical analysis is that the low detection response of some drugs can be



Salicylic Acid

$(CH_3CH_2CH_2)_2CH\ COOH$

Valproic Acid



Ibuprofen

greatly improved using counter-ions of high molar absorptivity or fluorescence intensity (5-8). While there have been literature reports on the analysis of amines using ion-pair extraction techniques (9-12), there have been few, if any, reports of using such techniques to improve the detectability of carboxylic acids on-line.

In this study, highly chromophoric and fluorophoric cationic dyes were evaluated as appropriate counter-ions for the ion-pair extraction of carboxylic acid-containing drug moieties in a flow injection system. The dyes studied were malachite green, thioflavin-T, methylene blue, gentian violet, acridine orange, and acridine red. The flowing stream system was designed such that a chloroform solution of the cationic dye was added to a predominantly aqueous mobile phase containing carboxylate ion post-injection and, after mixing, the chloroform containing the extracted dye-carboxylate ion-pair was separated from the mobile phase using a phase separator. Salicylic acid, ibuprofen, and valproic acid were selected as model carboxylic acid drugs to validate the flow-injection methods developed in this study. The method was also applied to the assay of a prostaglandin ($PGF_{2\alpha}$).

EXPERIMENTAL

Apparatus

Spectrophotometric and fluorometric spectra were obtained using either a Bausch and Lomb Model 2000 Spectrophotometer or

a Perkin-Elmer Model MPF-4 Spectrophotofluorometer. The HPLC system consisted of a Waters Associates Model 6000A pump, a Model U-6K injector, and either a Perkin-Elmer Model LC-5 UV/VIS detector or a Schoeffel Model FS-970 fluorometric detector. The UV/VIS detector was modified with a flow-through quartz cell containing inlet and outlet stainless steel tubing of 0.020 in. i.d. (Hellman Cells, Forest Hill, NY 11375). The cell in the fluorometer was also modified to contain 0.020 in. i.d. inlet and outlet tubing. Recordings were made using a Fisher Series 5000 strip-chart recorder set at 10 mV.

The on-line post-column ion-pair extraction system consisted of a Brinkmann Model 131900 peristaltic pump, a 30-turn glass mixing coil (Technicon, S/N 178/G196-04) and a glass phase separator (Technicon Type 1, S/N 021-B007-01) equipped with a small piece of teflon tubing cut lengthwise to aid in phase separation. Acidflex flow rated pump tubing (Technicon, S/N 116-0651 P13) was used to deliver the chloroform solution to the HPLC mobile phase. Nipple fittings (Technicon, S/N NP-116-001 P01) were used as connectors between stainless steel tubing, the glass mixing coils, the glass phase separator, and the UV/VIS or fluorescence detector.

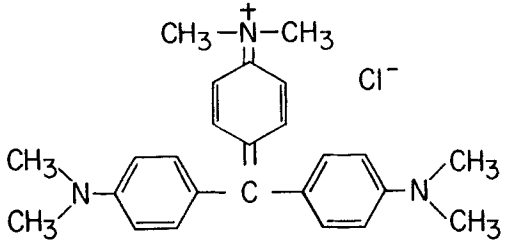
Chemicals and Reagents

Sodium salicylate was obtained from Baker Chemical Co. (Phillipsburg, NJ 08865). Valproic acid was a gift from Abbott

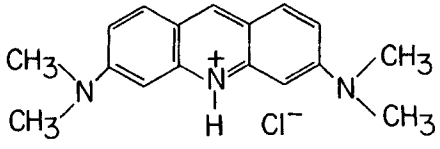
Laboratories (N. Chicago, IL 60064) and ibuprofen and prostaglandin ($PGF_{2\alpha}$) were supplied by the Upjohn Company (Kalamazoo, MI 49003). The following dyes and their respective suppliers were used: gentian violet (Fisher Scientific Co.), Malachite green (Hartman-Leddon Co.), thioflavin T, acridine orange and methylene blue (Spectrum Chemical Manufacturing Corp.), and acridine red (INC Pharmaceuticals). All other chemicals and solvents were of the highest grade commercially available.

Preparation of Stock Solutions

Stock solutions (1.95×10^{-5} M) of gentian violet, melachite green, thioflavin T, acridine red, acridine orange, and



Gentian Violet
(Triarylmethane)



Basic Orange 14
(Acridine)

methylene blue were prepared in chloroform that was saturated with 0.05 M phosphate buffer, pH 7. Stock solutions (100 µg/ml) of salicylic acid, valproic acid and ibuprofen and a stock solution (1 mg/ml) of prostaglandin ($PGF_{2\alpha}$) were also prepared in 0.05 M phosphate buffer, pH 7. Serial dilutions were made from all of the drug stock solutions for construction of calibration curves and for spiked samples.

Chromatographic Parameters

A mobile phase of 90:10 aqueous 0.01M phosphate buffer (pH 7)-absolute methanol was pumped at a flow rate of 1 ml/min. A 1.95×10^{-5} M solution of a dye in chloroform was pumped through acidflex tubing into the mobile phase at a flow rate of 1.25 ml/min using the peristaltic pump. The UV/VIS detector was set at the λ_{max} of the respective dye for the spectrophotometric evaluation and the fluorescence detector was set at the appropriate excitation wavelength of each dye with the employment of an emission filter >470 nm. Fifty microliter injections of analytical solutions containing the various concentrations of salicylic acid, valproic acid and ibuprofen were injected into the mobile phase using a 100 µl syringe. Replicate injections were made for each sample. To test the application of the flow injection ion-pair extractor in the separation mode, an octadecylsilane column (10 µm, 100 mm x 4.6 mm i.d.) was inserted after the injector but before the mixing

tee where counterion solution was added. Peak height data in mm was manually measured from each chromatogram and the data subjected to linear regression analysis. Slope, intercept and correlation coefficient values were calculated for each drug studied. The slope and intercept values were then used to calculate concentration of drug in spiked samples. Accuracy and precision data were also determined.

RESULTS AND DISCUSSION

This study investigated the use of VIS absorbing or fluorescent cationic dyes as ion-pair extraction reagents for carboxylic acid drugs in a flowing stream system. This involved designing a flow injection ion-pair extractor utilizing post-column reactor technology and applying this concept to the quantitation of drug-dye ion-pairs using either spectrophotometric or fluorescence detection. The VIS dyes investigated were gentian violet, malachite green, thioflavin T., and methylene blue for spectrophotometric evaluation and acridine red and acridine orange for fluorescence evaluation. The carboxylic acids selected as model drugs were salicylic acid, valproic acid, and ibuprofen.

There have been reports in the literature on the application of continuous flow ion-pair extractor systems for the detection of basic analytes eluting from a liquid chromatograph column (13-15). These extractor systems have

usually consisted of standard commercially available micro-glassware that have been assembled into numerous designs based upon the desired interaction of analyte and flowing stream (16,17). More recently, other investigators have reported the use of home made phase separators in which teflon coated membranes have been used to separate the aqueous and organic components of the mobile phase (18-20). Lawrence has also demonstrated the use of a hydrophobic solvent-inert filter disc loosely inserted into the detection outlet arm of a Y connector as an effective post-column phase separator (21,22).

The continuous-flow ion-pair extraction system designed for this study is shown in Fig. 1. It is based on a solvent-segmented design and utilizes a commercially available glass mixing coil and a glass phase separator (see Fig. 2) which were inserted into a typical flow injection analysis (FIA) system. Back pressure in the system was reduced through the use of 0.020 in. i.d. inlet and outlet tubing to the UV/VIS and fluorometric detectors. Solvent segmentation in a flowing stream allows for longer reaction times, provides a minimum amount of band broadening, and is less sensitive to temperature and flow variations than air-segmentation. Initially, FIA was employed in designing the method and evaluating the post-column ion-pair extractor system. Later, the applicability of using the system with an octadecylsilane column inserted on-line was demonstrated for ibuprofen.

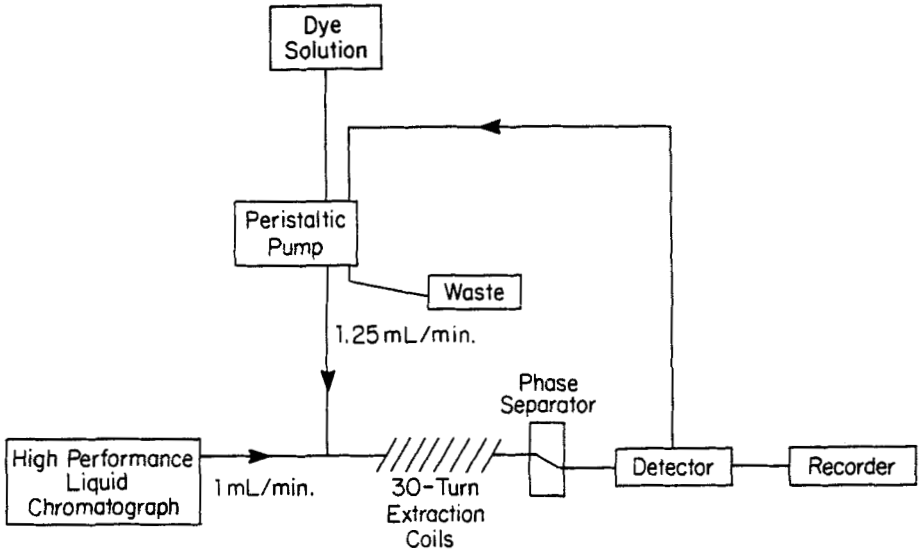


Figure 1 - Schematic of Flow Injection Ion-Pair Extractor System.

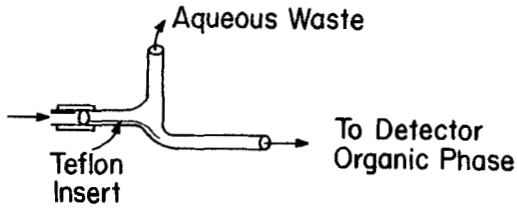


Figure 2 - Diagram of the commercially available phase separator used in the flow injection system.

Preliminary spectrophotometric and fluorometric experiments were performed to define the optimum ion-pairing parameters to use in the flowing stream system. Factors considered were selection of the best cationic dye(s) to serve as ion-pairing reagent(s) by determining optimum dye to drug ratios, selection of the best organic extraction solvent, selecting the optimum pH at which a particular acidic drug is extracted as an ion-pair, and performing stability studies on the extracted drug/dye ion-pair.

Hexane, carbon tetrachloride, methylene chloride, and chloroform were evaluated as extraction solvents for the dye-drug ion-pairs. The log extraction constants (E_{QX}) for the three model carboxylic acid drugs are shown in Table 1.

TABLE 1

Log Extraction Constants (E_{QX}) for Model Carboxylic Acids Using Gentian Violet as Counter-Ion[a]

Carboxylic Acid	Log E_{QX}			
	Hexane	Carbon Tetrachloride	Methylene Chloride	Chloroform
Salicylic Acid	2.89	3.96	4.79	5.30
Valproic Acid	2.96	4.15	4.52	5.01
Ibuprofen	3.33	3.97	4.28	4.79

[a]Four ml of 7.8×10^{-5} M gentian violet in each organic solvent was mixed with 4 ml of 1.95×10^{-5} M drug solution in pH 7 phosphate buffer. After separation, the absorbance of the organic layer was measured at 588 nm and and log E_{QX} calculated.

Chloroform was shown to be a slightly better extraction solvent based on the data and, therefore, it was selected for use in this study.

Dye to drug ratios in the ranges 1:1 to 200:1 were studied using salicylic acid as the model compound. Results indicated that only gentian violet and malachite green among the VIS absorbing dyes showed a constant percent extraction of salicylic acid over 4:1 to 100:1 ranges. For the fluorescent dyes, the dye-drug ratios were studied in the 1:1 to 10:1 ranges. Only acridine orange gave a constant percent extraction for salicylic acid over 4:1 to 10:1 ranges. Therefore, the concentration of either VIS or fluorescent dye in the flowing stream was adjusted such that at least a 4:1 excess of dye to drug was present.

The optimum pH for extraction of the dye-drug ion-pair was determined for all three model compounds using chloroform as extraction solvent and gentian violet as counterion. It was found that maximum extraction of the ion-pair occurred in the pH 7-9 range for all three carboxylic acids. Because bonded phase HPLC columns have pH restrictions, all of the drug studies were performed at pH 7.

Using ion-pairs formed with salicylic acid and either gentian violet, malachite green, or acridine orange, the ion-pairs formed with gentian violet and acridine orange gave very stable absorbance and fluorescence readings in chloroform for 30 min after extraction. Malachite green showed a

significant decrease in absorbance over time. Therefore, it was decided that gentian violet would be the counter-ion of choice for the acidic drugs in the spectrophotometric study and acridine orange would be the choice in the fluorescence study.

Molar ratio binding data for both gentian violet and acridine orange with salicylic acid indicated that there was a one to one binding ratio between dye and the drug. Additional experiments in this laboratory confirmed that the VIS absorption maxima and the fluorescence excitation and emission wavelengths for the ion-pairs were identical to that of the dye being used as the counter-ion.

Then, linearity studies were performed on-line using the continuous flow ion-pair extractor HPLC system designed for this study. A mobile phase of 90:10 aqueous phosphate buffer (pH 7) - absolute methanol was pumped at a flow rate of 1.0 ml/min. A chloroform solution saturated with pH 7 phosphate buffer and containing either gentian violet or acridine orange was pumped into the mobile phase. Each acidic drug was injected into the FIA system as a 50 µl injection. Typical chromatograms for ibuprofen and valproic acid using spectrophotometric detection are shown in Fig 3. The linear concentration range of the procedure and regression analysis of the gentian violet-drug data are shown in Table 2. Accuracy and precision were calculated to be in the 2-6% and 3-7% ranges, respectively, for spiked samples run as unknowns (see Table 3).

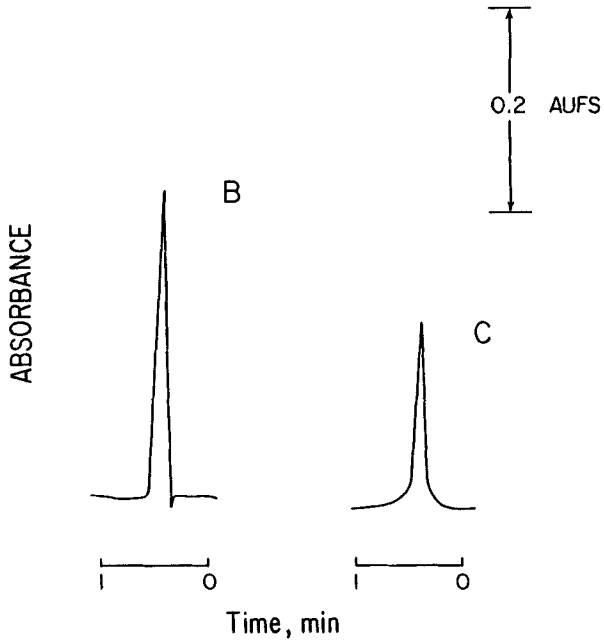


Figure 3 - Typical FIA chromatograms of 5 µg/ml ibuprofen (B) and 5 µg/ml valproic acid (C) using the flow injection ion-pair extractor system with gentian violet as counterion and detection at 588 nm. Sample injection volume was 50 µl.

The acridine orange data indicated that two to four fold increases in sensitivity were obtained for the fluorescence versus spectrophotometric detection for all the drugs studied. Typical chromatograms for salicylic acid, ibuprofen, and valproic acid using fluorescence detection are shown in Fig. 4. The linearity of the method and linear regression data for each acid are shown in Table 4. Accuracy and precision data for the assay using spiked samples of the drugs are shown in Table 5.

TABLE 2

Linear Regression Analysis of Peak Height - Absorbance Data for Acidic Drugs Using the Flow Injection Ion-pair Extractor with Gentian Violet as Counter-Ion

	Salicylic Acid	Valproic Acid	Ibuprofen
Linear concn range, µg/ml	0.5 - 10	5 - 50	1 - 10
Intercept	1.678	1.678	2.315
Slope	0.746	0.746	6.120
Correlation Coefficient	0.9954 (n = 10)	0.9964 (n = 15)	0.9920 (n = 11)

[a]Measured at 588 nm.

TABLE 3

Accuracy and Precision Data for Spiked Samples of Model Carboxylic Acids Using Gentian Violet with Spectrophotometric Detection

Compound	Concn added, µg/ml	Concn found, µug/ml[a]	Accuracy, %	RSD,%
Salicylic Acid	1.0	1.03 ± 0.059	3.0	5.7
	5.0	5.12 ± 0.167	2.4	3.3
Valproic Acid	15.0	14.51 ± 0.670	3.3	4.6
	30.0	28.92 ± 1.280	3.6	4.4
Ibuprofen	2.5	2.45 ± 0.125	2.0	5.1
	4.0	3.76 ± 0.249	6.0	6.6

[a]Based on triplicate determinations measured at 588 nm.

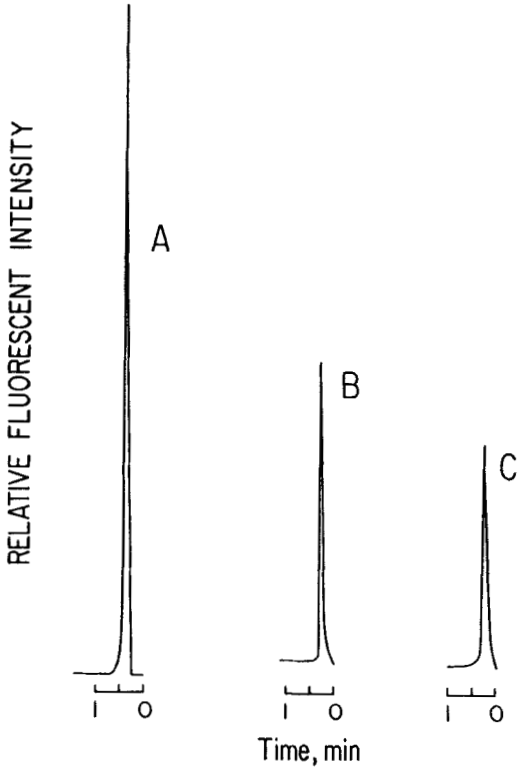


Figure 4 - Typical FIA chromatograms of 5 µg/ml salicylic acid (A), 10 µg/ml ibuprofen (B), and 25 µg/ml valproic acid (C) using the flow injection ion-pair extractor system with acridine orange as counterion and fluorescence detection using λ_{ex} 297 nm and an emission filter > 470 nm. Sample injection volume was 50 µl.

There was interest in this laboratory in the application of the procedure to the analysis of a prostaglandin ($PGF_{2\alpha}$). Linear regression data using gentian violet as ion-pair reagent revealed that peak height was linear with concentration in the 16-500 µg/ml range. Slope, intercept and correlation

TABLE 4

Linear Regression Analysis of Peak Height - Fluorescence Data for Acidic Drugs Using the Flow-Injection Ion-Pair Extractor with Acridine Orange as Counter-Ion[a]

	Salicylic Acid	Valproic Acid	Ibuprofen
Linear concn range, µg/ml	0.13 - 5	2.5 - 50	0.5 - 20
Intercept	0.381	1.082	1.130
Slope	37.640	1.922	5.996
Correlation Coefficient	0.9980 (n = 10)	0.9994 (n = 9)	0.9989 (n = 10)

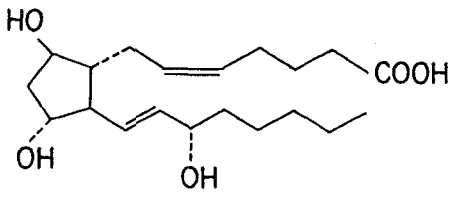
[a]Measurements were made using an excitation maximum of 297 nm and an emission filter > 470 nm.

TABLE 5

Accuracy and Precision Data for Spiked Samples of Model Acidic Drugs Using Acridine Orange and Fluorescence Detection

Compound	Concn added, µg/ml	Concn found, µg/ml[a]	Accuracy, %	RSD, %
Salicylic Acid	0.25	0.24 ± 0.013	4.0	5.4
	3.0	2.99 ± 0.038	0.3	1.3
Valproic Acid	5.0	5.03 ± 0.184	0.6	3.7
	25.0	25.06 ± 0.552	0.2	2.2
Ibuprofen	1.0	1.03 ± 0.048	3.0	4.7
	10.0	10.15 ± 0.333	1.5	3.3

[a]Based on triplicate determinations measured at λ_{ex} 297 nm and an emission filter > 470 nm.



$PGF_{2\alpha}$

coefficient data were 0.164, 1.432 and 0.9973 (n = 12), respectively. Spiked samples of $PGF_{2\alpha}$ containing 31.3 and 125 µg/ml levels were analyzed as unknowns. Based on regression data, it was calculated that the concentration of prostaglandin in these samples were 31.6 ± 2.9 and 128.3 ± 7.9 µg/ml (n = 4). This data indicated that the method would provide an accuracy of 1-3% and precision of 5-6% for a prostaglandin.

The flow injection system was also applied to the assay of commercial dosage forms containing valproic acid and ibuprofen. The results of the assays are shown in Table 6. The data indicates that the FIA method developed herein is capable of providing good recovery of drug from a dosage form matrix.

Then, an octadecylsilane column was inserted into the FIA system. Ibuprofen was injected into the 90:10 phosphate buffer-methanol mobile phase with gentian violet employed as the counter-ion. The retention time of the drug was found to be 2 min using a flow rate of 1 ml/min. Peak height was linear with concentration in the 2.5 - 40 µg/ml range. Slope, intercept and correlation coefficent data were 0.514, 1.143 and 0.9982 (n =

TABLE 6

Assay of Valproic Acid and Ibuprofen Dosage Forms Using the Flow Injection Ion-pair Extractor System[a]

Dosage Form	Labeled claim, mg	Concn Found, mg	% of labeled claim
Valproic Acid[b]	250	243.7 ± 9.6 (n = 5)	97.5
Ibuprofen[c]	400	394.6 ± 13.6 (n = 6)	98.7

[a]Gentian violet was used as counterion and measurements were made at 588 nm.

[b]Depakene capsule, Abbott Labs.

[c]Motrin tablet, Upjohn Company.

12), respectively. Data from spiked ibuprofen samples analyzed at the 5 and 10 µg/ml level are shown in Table 7.

In summary, this study has shown that either visible absorbing or fluorescent cationic dyes can be used as counterions in the extraction of carboxylic acids in a flowing stream system. The long range ramifications of using these dyes in the analysis of non-UV/VIS absorbing or non-fluorescent carboxylic acids could provide a new means of detection for drugs containing this functionality. Studies are presently underway in this laboratory to improve the design and efficiency of the post-column mixing coils and phase separator.

TABLE 7

Analysis of Spiked Ibuprofen Samples Using an Octadecylsilane Column and the Ion-Pair Extractor With Gentian Violet as Counter-Ion[a]

Concn added, µg/ml	Concn found, µg/ml[b]	Accuracy, %	RSD, %
5.0	5.09 ± 0.253	1.8	4.97
10.0	9.91 ± 0.252	0.92	2.54

[a]Measurements were recorded at 588 nm.

[b]Based on n = 9.

ACKNOWLEDGEMENT

J. R. Lang's present address is Glaxo, Inc., Research Triangle Park, NC 27709.

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