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An Automated Continuous-Flow System for the Extraction and Fluorometric Analysis of Histamine

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The automated continuous-flow system for the extraction and fluorometric analysis of histamine is accurate, specific, precise, rapid, and very sensitive. The method was made possible by the use of a simple connector for separating the organic–aqueous phases at two steps in the procedure. Samples with and without protein can be analyzed successfully. The method can quantitate as little as 0.5 ng of histamine base.

The extraction and assay of histamine by a fluorometric method is a laborious but accurate method for the determination of this biologically active amine. The crucial role of histamine in allergic reactions has been well-documented. An automated system of histamine analysis could therefore facilitate *in vitro* studies of allergy and mechanisms of histamine release from cells.

The fluorometric method was originally described by Shore *et al.* (1) and has been modified since then to increase both its specificity and sensitivity (2, 3, reviewed in 4). The method is based on the coupling of histamine with *o*-phthalaldehyde at a highly alkaline pH to form a fluorescent product. The fluorescence of the histamine–*o*-phthalaldehyde complex is more intense and more stable at an acid pH, unlike the complex formed by some other amines. To remove other interfering compounds, the histamine is extracted prior to the condensation step. Protein is removed from the sample to be analyzed by perchloric acid precipitation; the histamine is extracted into *n*-butanol from the alkalinized salt-saturated solution. The histamine is recovered in an aqueous solution of dilute HCl by adding heptane. This dilute HCl solution is then used for the condensation of histamine with *o*-phthalaldehyde. The extraction procedure is essential to remove histidine and other interfering compounds prior to the condensation step.

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Previous attempts to automate the analysis of histamine involved only one of these steps, the condensation step (5) or partial extraction of the histamine (6). In the method described by Ruff *et al.*, the histamine is extracted into butanol and the condensation product formed in that solvent (6). Several compounds including histidine will interfere with the reaction. In contrast, the method presented in this paper is an automation of the entire procedure. It was made possible by the design of a simple and efficient extractor for the separation of the aqueous and organic phases at two steps during the assay.

EXPERIMENTAL

Apparatus. The following standard Technicon modules (Technicon Corp., Tarrytown, NY 10591) were used: sampler type II, two pumps type I, a dialyzer (temperature controlled at 37°C), and a single pen recorder. A Turner model 110 filter fluorometer with automated chemistry adapter was used, and excitation was achieved with the general-purpose mercury lamp (4 W). The filter systems included a narrow-pass primary filter 7–60 (peaks at 350 nm) and, secondarily, a sharp-cut filter 2A (415 nm) alone or in combination with filter 4–72 (Corning Laboratory Products). In measuring the parameters of the system, a strip chart recorder (Heath EU-205) was attached to the fluorometer.

Reagents. All the chemicals were reagent grade. Saline is prepared by dissolving 9.0 g of sodium chloride and 0.5 ml of Brij-35 (30% solution, Technicon Corp.) per liter; the solution is made 1.5×10^{-3} M with EDTA (stock solution of 0.1 M, pH 7.4, is used). A 30% sodium chloride solution is prepared by dissolving 300 g/liter. The 5 N NaOH is made from a 50% solution by diluting 26 ml up to 100 ml; the 1 N NaOH is prepared from a 10 N solution and contains EDTA at a final concentration of 1.0×10^{-3} M. The *o*-phthalaldehyde is recrystallized from ligroin and prepared as a 1 mg/ml solution in methanol (spectrophotometric grade) and stored in a brown bottle. It should be made up in small amounts enough for several days and when not in use stored at 4°C. The 0.1 N HCl is prepared from a 1 N commercial solution. Phosphoric acid (0.73 M) is made from an 85% solution by diluting 50 ml up to a liter. The 1-butanol (butyl alcohol) was reagent grade; the *n*-heptane was either fluorometric grade or regular laboratory grade with no appreciable difference in the results.

Standards. Histamine dihydrochloride was dissolved in 0.1 N HCl solution. The concentration of histamine is stated for the base. The use of standards prepared in saline, HCl, or 2% perchloric acid gave identical results. Stock standards were therefore kept in perchloric acid.

Procedure. The flow diagram for the automated histamine analysis is

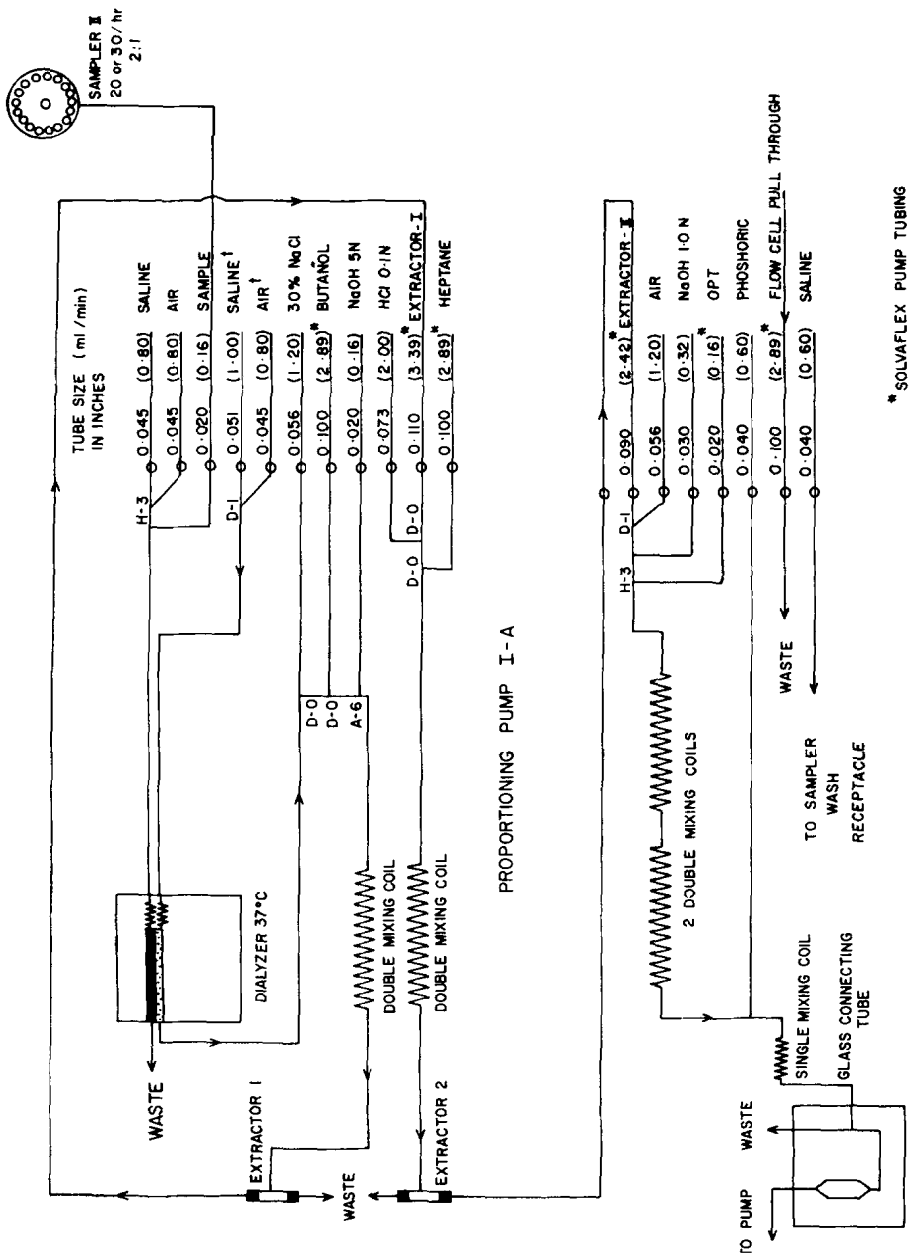
shown in Fig. 1. The procedure requires two pumps; the flow rates and tubing diameters are given in the figure. The histamine sample is diluted with saline containing Brij and EDTA and then dialyzed against the same solution. After dialysis, 30% NaCl and 1-butanol are added, and the stream is alkalinized to extract the histamine into butanol. In the double mixing coil, there is rapid separation into discrete segments of aqueous and organic phases. The organic phase with the air bubbles is removed by the first extractor from the upper arm. After repumping, 0.1 N HCl and *n*-heptane are added and the stream mixed. The organic–aqueous phases are separated in the second extractor; the aqueous lower phase is removed and resegmented with air. It is then alkalinized, *o*-phthalaldehyde added, and after the optimal reaction time, the histamine–*o*-phthalaldehyde reaction is stopped with phosphoric acid. The stream then passes through the fluorometer. The total time required for samples to run through the instrument is about 16 min.

Both standard and Solvaflex tubing are used as indicated in Fig. 1. Some of the volumes appear to be incorrect (i.e., the amount of aqueous sample taken from extractor II by the second pump is larger than the amount of HCl pumped in by the first pump). However, these sizes have been found to work well. There might be slight differences in the speed of the two pumps. If the second extractor does not exclude the organic phase completely, then a smaller pull-through should be used to resample the aqueous phase from the second extractor.

The length of the tube from extractor II to the pump should be as short as possible as this portion is not segmented by air. For accurate results, the end of the waste tubes from the extractors should be kept at the same level as the fluid level in the middle of the extractor, otherwise a negative pressure will be applied in part of the manifold and produce uneven flow characteristics.

The flow system can be modified to increase the sensitivity when analyzing histamine in samples which contain little or no protein ("high sensitivity" system). The changes required in the manifold are the following: the sample is diluted with saline as in the regular system; after passing through a single mixing coil, it bypasses the dialyzer after which point all connections are identical (Fig. 1). The total time required for samples to run through this manifold is about 12 min.

Extractor. A special extractor had to be designed to separate the aqueous from the organic phase at two steps in the automated procedure. The extractors are constructed by using the following Technicon manifold parts: a wide-bore connector (D-3) and two nipples (N-6). The two nipples should fit snugly inside the wide arms of the connector (Fig. 2). The diameter of the wide side arms should be close to 4 mm, and only


SAMPLER II
20 or 30/hr
2:1
TUBE SIZE (ml/min)
IN INCHES
H-3 0·045 (0·80) SALINE
0·045 (0·60) AIR
0·020 (0·16) SAMPLE
D-1 0·051 (1·00) SALINE†
0·045 (0·80) AIR†
0·056 (1·20) 30% NaCl
0·100 (2·89)* BUTANOL
0·020 (0·16) NaOH 5N
0·073 (2·00) HCl 0·1N
D-0 0·110 (3·39)* EXTRACTOR-I
0·100 (2·89)* HEPTANE
WASTE
EXTRACTOR 1
DIALYZER 37°C
WASTE
EXTRACTOR 2
D-0
D-0
A-6
DOUBLE MIXING COIL
DOUBLE MIXING COIL
PROPORTIONING PUMP I-A
H-3 D-1 0·090 (2·42)* EXTRACTOR-II
0·056 (1·20) AIR
0·030 (0·32) NaOH 1·0 N
0·020 (0·16)* OPT
0·040 (0·60) PHOSPHORIC
0·100 (2·89)* FLOW CELL PULL THROUGH
0·040 (0·60) SALINE
2 DOUBLE MIXING COILS
WASTE
TO SAMPLER WASH RECEPTACLE
SINGLE MIXING COIL
GLASS CONNECTING TUBE
TO PUMP
WASTE
FLUOROMETER
PROPORTIONING PUMP I-B
*SOLVAFLEX PUMP TUBING

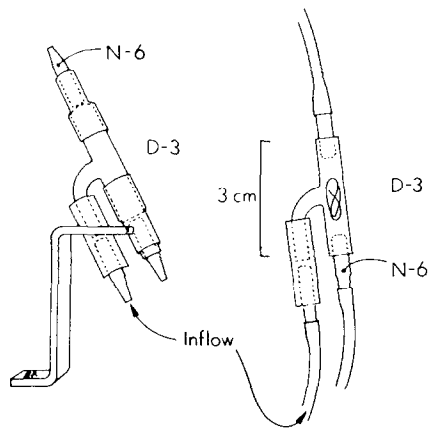


FIG. 2. Extractors used to separate organic–aqueous phases. The diameter of the wide side arms of the D-3 connector should be at least 4 mm. Either one of these two extractors shown has been found to function well.

connectors with this size side arms are used as extractors. The aqueous organic mixture to be separated flows in the narrow side arm with the extractor set vertically or at an angle of 110° from the horizontal. Depending on the stage of the extraction, either the aqueous or organic phase is removed for further processing. The height of the two nipples can be adjusted to obtain the optimal volume in the extractor. The introduction of a 5-cm coiled thin steel wire in the extractors will break up small bubbles and improve the separation of the two phases.

Performance. The performance of the manifold was assessed by the use of histamine standards and the development of a plateau with the probe in a sample for 10 min. The rise and fall curves were recorded on a strip chart recorder (at 5 sec/in.) and then used for the calculations of half-wash time, the lag phase, the value of a and b according to the method of Thiers *et al.* (7) and Walker *et al.* (8). The lag phase was also calculated by the method described by Strickler *et al.* (9).

RESULTS

Samples. The amount of sample required is about 0.5 ml, although the actual volume analyzed will depend on the rate of analysis. The system with and without dialysis has been tested with samples contain-

FIG. 1. Flow diagram for histamine analysis. The first and second pumps have 5 and 10 rollers, respectively. Manifold is for "low sensitivity" protein-containing samples and includes dialysis. "High sensitivity" for low-protein samples is achieved by removing the tubes marked (+) and replacing the dialyzer with a single mixing coil.

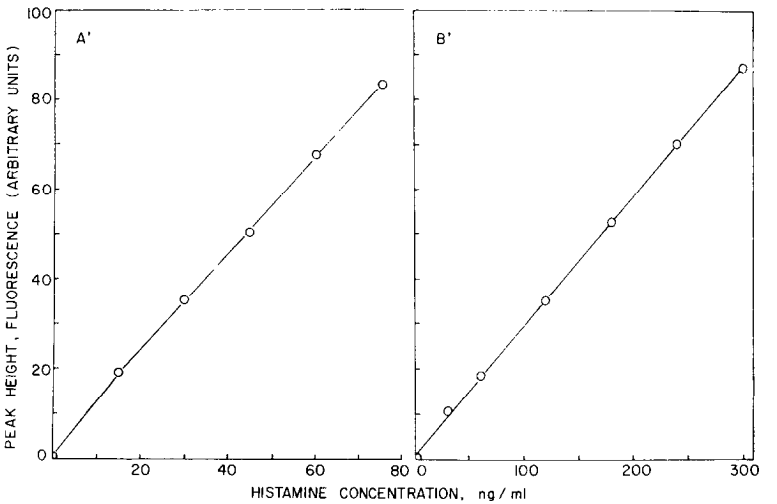


FIG. 3. Calibration curve for histamine standards analyzed with dialysis ("low sensitivity") (A') Standards 0–80 ng/ml; (B') standards 0–300 ng/ml. Analysis at 20 samples/hr; fluorometric settings changed between the two sets. Histamine standards were in 2% perchloric acid. Solutions used on manifold as described under *Experimental*.

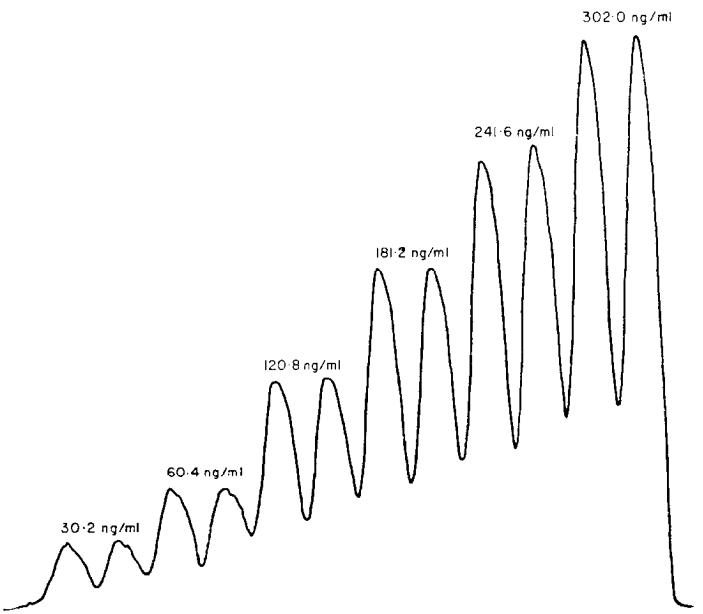


FIG. 4. Strip-chart recording of histamine standard curve shown in Fig. 3 B'.

ing different amounts of protein. With dialysis, histamine could be analyzed and was recovered quantitatively when added to different amounts of serum up to 90% serum. When human serum albumin, up to concentrations of 80 mg/ml, was added to histamine standards in saline, there was no interference with the analysis or the recovery of histamine. In contrast, when the manifold was utilized without the dialyzer, samples could not be analyzed if they contained more than 3.0 mg of albumin/ml. At higher protein concentrations, the results became erratic due to protein precipitation and collection in the first extractor.

Extractors. The two extractors function efficiently. There is minimal loss of the organic or aqueous phases in the respective extractors (15% and 23%, respectively). Furthermore, there is no flow through of the excluded phase, i.e., in extractor I, no aqueous phase passes through with the butanol, and in extractor II, no organic phase with the 0.1 *N* HCl. Within the extractors, the different phases form discrete levels, and any small bubbles are broken up by the thin steel wires. The use of beaded-glass mixing coils in the manifold at both extraction steps (after the addition of butanol and after the addition of heptane) did not improve the efficiency of the histamine extraction. Phase separation did not improve by the addition of settling coils preceding the extractors. A number of different connectors were tried as extractors; none gave uniformly good results except the one described and shown in Fig. 2.

Sensitivity. When analyzing samples with high protein concentrations,

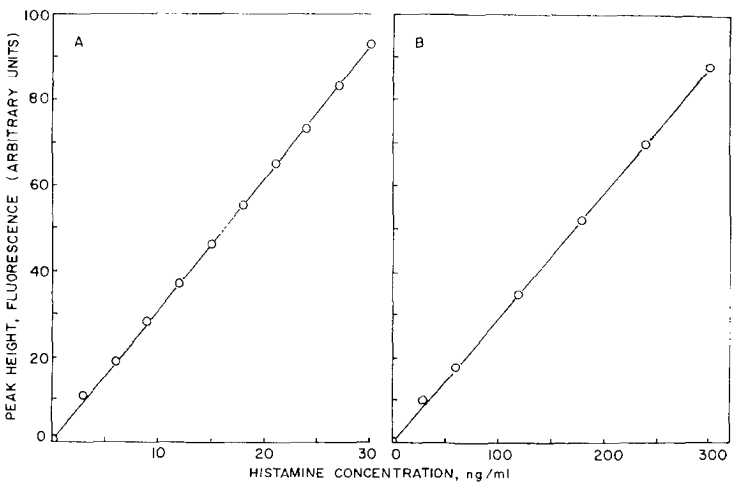


FIG. 5. Calibration curve for histamine standards analyzed without dialysis ("high sensitivity"). (A) Standards 0–30 ng/ml; (B) standards 0–300 ng/ml. (See Fig. 3 legend for details.)

the maximum sensitivity of the system is 100 ng/ml for full-scale deflection on the recorder. Figure 3 illustrates the results obtained when standards are analyzed with dialysis in the range of 20 to 80 ng/ml and from 50 to 300 ng/ml. The actual recorder readings for the second set is also shown by the photograph in Fig. 4. The sensitivity of the manifold is greatly increased by bypassing the dialysis when analyzing samples with low protein content. The range of maximum sensitivity that can be obtained is shown by the results in Fig. 5, where samples contain from 1 to 30 ng/ml. As the total amount of sample analyzed is only 0.5 ml,

TABLE 1
Interference of Compounds with the Automated Histamine Assay

Compound[a]	Molar fluorescent ratio[b]	Interference[c] (%)
Spermidine	105	−2.3
Spermine	1,400	−6.7
Chloroquine	5,620	0
Agmatine	6,800	+2.2
Histidine	8,470	+5.0
N-Acetylhistamine	8,990	+11.1
Colchicine	17,000	+1.3
Imidazole	21,900	+1.2
Serotonin	24,400	−1.0
Imidazole acetic acid	No fluorescence	+3.5
Urocanic acid		−0.3
L-Carnosine		+1.3
Methionine		+1.3
Valine		+2.5
Alanine		+2.1
Glutamine		−0.3
Phenylalanine		−4.8

[a] All compounds were analyzed as 1 mg/ml solutions ($2–14 \times 10^{-3}$ M) except for spermidine, which was tested at a concentration of 0.1 mg/ml.

[b] Histamine standards and the solutions were analyzed by the continuous-flow system.

$$\text{Molar fluorescent ratio} = \frac{\text{Molar fluorescence of histamine}}{\text{Molar fluorescence of compound}}.$$

The ratio is a measure of the selectivity of the steps in the analysis procedure.

[c] Equal volumes of a histamine standard (600 ng/ml) and the solution of the compound (1 mg/ml) were mixed and analyzed. Interference is for the whole assay system.

$$\text{Interference} = \frac{A - B - C}{A + B + C} \times 100.$$

A = Fluorescence of histamine-compound solution.
B = Fluorescence of histamine standard (300 ng/ml).
C = Fluorescence of a solution of the compound alone (0.5 mg/ml).

the limit of detection for this system is about 250 pg of histamine base. The sensitivity of the system can be further increased by the use of a more powerful fluorometer.

Interfering compounds. A large number of compounds, especially some amines, can react with *o*-phthalaldehyde to give a fluorescent complex (10). However, the extraction procedure and the reaction conditions are such as to exclude most of these compounds. To further test the system, a number of compounds were tested either alone to measure the amount of fluorescence or with a constant amount of histamine to see their effects on the extraction steps or their possible interference in the histamine-*o*-phthalaldehyde complex. The manifolds were tested with and without dialysis with comparable results (Table 1). The molar ratios of most of these compounds required to give the same fluorescence as histamine are very large. Spermidine, if present in samples, is extracted and causes fluorescence (molar fluorescent ratio of 105); spermine is less active. Most other compounds had molar fluorescent ratios over 5,000. The effect of adding these compounds to histamine was quite similar; spermidine, spermine, and acetylhistamine inhibited the fluorescence of histamine most actively. Other compounds had minimal effects.

Performance. The parameters of the system are given in Table 2. The complexity of the manifold results in the high half-wash time ($W_{1/2}$). The use of extractors with their mixing volumes and the unsegmented lines are other reasons that the system has these high values.

Accuracy and precision. Recovery experiments were performed using histamine in saline solution. The difference between the calculated and measured results is shown in Table 3. The correlation coefficient between

TABLE 2
Parameters of the Automated Continuous-flow Histamine Analysis System[a]

Parameter	With dialysis	With no dialysis	Reference
Half-wash time ($W_{1/2}$)	0.41 min	0.37 min	7
Lag time (L or *a*)	0.46 min	0.33 min	7, 8
b	0.58 min	0.53 min	8
Carry over at			
20/hr 2:1	1.0% (0.6%)[b]	1.0% (0.4%)[b]	8
30/hr 2:1	5.3% (3.2%)[b]	4.4% (2.3%)[b]	
Peak height as percentage of plateau			
20/hr 2:1	93% (97%)[b]	97% (98%)[b]	
30/hr 2:1	84% (90%)[b]	86% (92%)[b]	

[a] The sample probe was placed in a histamine solution and a strip-chart recording made of the rise and fall curves. Calculations were as described in (7–9). All solutions used were those described in *Experimental.*

[b] Theoretical values calculated using *a* and *b*. (8).

TABLE 3
Accuracy of the Histamine Analysis System

Histamine added[a] (ng/ml)	Histamine measured[b]							
	With dialysis				Without dialysis			
	N	Mean (ng/ml)	Standard deviation (ng/ml)	Relative standard deviation (%)	N	Mean (ng/ml)	Standard deviation (ng/ml)	Relative standard deviation (%)
30.2	5	27.7	0.4	1.4	6	30.5	0.7	2.3
60.4	4	60.6	1.0	1.7	7	60.7	0.6	1.0
90.6	5	91.7	0.7	0.8	5	91.2	1.2	1.3
120.8	4	120.6	0.7	0.6	5	121.8	0.3	0.2
151.0	5	152.0	0.8	0.5	5	152.9	1.9	1.2
302.0	4	299.9	2.1	0.7	4	296.6	2.7	0.9

[a] These histamine standards in saline were added to solutions containing 0, 15.1, 30.2, 60.4, 120.8, and 241.6 ng histamine/ml. The histamine concentration of these solutions were then determined.

[b] The coefficient of correlation (r) for all the values is 0.99. The regression line is $\alpha = 1.36$, $\beta = 0.99$.

the two results was 0.99; the relative standard deviation at all levels was in the range of 0.2 and 2.3%.

The precision of the method was determined by repeated analyses of samples. The reproducibility was satisfactory in both systems as shown by the results in Table 4. The relative standard deviation is about 1%. There is some problem of machine drift. If the system is used for 5–7 hr,

TABLE 4
Precision of the Histamine Analysis System[a]

Manifold used[b]	Histamine concentration (ng/ml)	N	Range of peak heights	Mean[c]	Standard deviation[c]	Relative standard deviation
With dialysis	120	39	91.5–95.5	93.8	0.93	0.99
	300	40	86.0–89.5	88.0	0.74	0.84
With no dialysis	30	32	81.0–84.0	82.4	0.89	1.08
	300	40	86.0–90.0	88.1	0.98	1.1

[a] Histamine standard solutions in perchloric acid were analyzed repeatedly with or without dialysis. All solutions used were those described in *Experimental.*

[b] Analysis at the rate of 20/hr 2:1.

[c] Relative units; amplification settings of the fluorometer changed between different sets.

standards will occasionally decrease by about 10%. This can easily be corrected by inserting reference solutions every 40 samples.

Correlation with the manual extraction and fluorometric analysis. When 100 samples were analyzed both manually and by the automated method, the results were found to be very closely correlated ($r = 0.983$). The variation which is usually noted among duplicates when analyzed manually (about 5%) is not seen with the automated technique. The parameters of the regression line were $\beta = 1.072$ and $\alpha = -6.8$.

DISCUSSION

The automated continuous-flow histamine analysis system is capable of analyzing samples at the rate of 20–30/hr with a high degree of sensitivity, accuracy, and precision. The amount of sample required for analysis is also very small. In comparison, the manual extraction and histamine analysis requires a large amount of technical time: it takes a technician a full 7-hr work day to analyze 60 samples.

Studies of the parameters of the procedure allow certain predictions to be made. When samples are analyzed with dialysis at the rate of 30/hr, there is a carry-over of 3.2%; this decreases to 0.6% at the rate of 20 samples/hr. Without dialysis, the results are somewhat better: there is a carry-over of 2.3% at 30/hr and 0.4% at the rate of 20/hr. In most experimental studies where the difference between the amount of histamine in the blanks and experimental tubes is as much as 30-fold, the samples are analyzed at the rate of 20/hr.

The automated histamine method has been used routinely in this laboratory for the last 2 years in studies of histamine release from cells during allergic reactions. In experiments with rabbit platelets, there is 10% plasma in the reaction mixture and the samples are analyzed with dialysis. The automated histamine assay system has been used as a very reliable *in vitro* tool in the diagnosis of human allergies. Different allergens are added to whole blood and after an incubation at 37°C for 1 hr, the reaction mixtures are centrifuged. The supernatants which contain about 50% plasma are used for histamine assays directly on the analyzer. The total histamine content of whole blood from 50 patients was found to be in the range of 39–209 ng/ml (mean 83 ng/ml). The manifold without the dialyzer has also been used in the analysis of supernatants of reaction mixtures containing washed human or rabbit leukocytes where there is minimal amounts of protein in the samples.

It is recommended that the routine analysis of histamine samples include the dialysis step. This functions very well for samples whose total histamine content is 75 ng/ml. The system without dialysis should

be reserved for samples with little or no protein and small amounts of histamine (less than 25 ng/ml).

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