A RELIABLE COLORIMETRIC METHOD FOR THE QUANTITATIVE DETERMINATION OF PROCAINE IN HUMAN WHOLE BLOOD[1]

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A REVIEW of the literature on the estimation of procaine in blood reveals that many methods have been advocated for the quantitative estimation of this local anaesthetic (1, 2, 3, 4, 5). In Table I are listed the results of some investigations that have been reported. As one studies the methods that have been published it becomes apparent that the investigators did not appreciate the rapid enzymatic destruction of procaine in human blood (6, 7, 8, 9), the adsorption of procaine on precipitated blood proteins, and the alkaline hydrolysis of procaine in solution extracted for analysis (7, 10, 11).

TABLE I

BLOOD AND PLASMA LEVELS OF PROCAINE REPORTED IN THE LITERATURE

Species	Route	Amount	Procaine (mg /100 ml blood)	Reference
Dog	I V †	15 mg /kg (309 mg , 100 mg./min)	1 25 (at 2 min , 0 at 1 hr.)	12
	I V	1 5–2 5 mg /kg./min	4	13
Guinea pig	I V	45 mg /kg (lethal dose)	11 5	20
	I.P †	887 mg /kg (lethal dose)	46 3	
	I M †	375 mg /kg (lethal dose)	11 0	
	S C †	⟨75 mg /kg (lethal dose)	15 7	
	S C	622–755 mg /kg.	8 7–11 1 (fatal in 2)	2
		96–258 mg /kg	3 0–4 6 (toxic in 7)	
		12 1–50 6 mg /kg	0 65–1 95 (non-toxic)	
Rabbit	S C	200 mg.	1 2 (50–70 min)	14
	I V	20 mg /kg	32 (0 at 30 min)	3
	I V	30 mg /kg	0 9–0 2 (5–240 min)	15
		15 mg /kg	0 2–0 1 (15–240 min)	
	I P	60 mg /kg	0 9 (max. at 60 min)	
		30 mg /kg.	0 24 (max at 60 min)	
Cat	I V	0 5–2 5 mg /kg./min.	6–66 mg /l (0 at 30 min)	18
Human	Spinal	300 mg	0 016	1
	S C	2 27–13 6 mg /kg	0 2–0 3 (2/13)	2
	I V	200–500 mg./25 min	5 5–8 6	3
	I V	5 gm./120–405 min	0 28–1 2 (0 at 1 hr)	5
	I.V.	2 gm /45–125 min	Less than 0 35 mg /l * (0 at 1 hr.)	4

*Procaine mg /L. plasma.
†I V = intravenous, I P = intraperitoneal; I.M = intramuscular, S C = subcutaneous.

INACTIVATION OF SERUM CHOLINESTERASE

It has been recognized for at least ten years that the serum esterase in human blood rapidly destroys procaine; this rapid destruction does not hold true for blood of most laboratory animals—monkeys and some of the apes being exceptions

159

Can Anaes. Soc J , vol. 6, no. 2, April, 1959

(7, 9). The optimum velocity of hydrolysis by human serum esterase alone has been estimated to be 19 mg. procaine hydrochloride per minute (8). Therefore, any method devised for the estimation of procaine should involve the arrest of enzyme activity immediately upon withdrawal of a blood sample. Methods for inactivating the enzyme have been reported by many authors (1, 3, 5, 12, 13, 14, 15), some of whom followed the Bratton-Marshall method for sulfonamides (16); others (2, 7) have added trichloroacetic acid to the blood immediately upon its removal, thus destroying the enzyme and precipitating the blood proteins. Other chemicals such as sodium arsenite (4), sodium fluoride (11, 17), and sodium fluoride–potassium oxalate solution (18) have been used to advantage to inhibit enzyme activity without precipitating proteins. It was apparent from the literature that when the sodium fluoride content of human whole blood was M/2, enzymatic procaine hydrolysis was negligible; M/20 potassium oxalate was employed in blood samples as an anticoagulant (11).

EXTRACTION OF PROCAINE

Each of the workers who adopted the methods listed above, regardless of whether enzyme inhibition was first accomplished or not, employed trichloro-acetic acid (15–20 per cent) to precipitate the blood or plasma proteins without, mentioning the possibility of procaine being hydrolysed by the acid. In order to test whether such hydrolysis occurred, trichloroacetic acid solutions (20 per cent) containing 0.2 mg. procaine hydrochloride or 0.1 mg. PABA were measured in the DK Spectrophotometer for any change in concentration and as none was apparent, it was assumed that acid hydrolysis was absent.

The filtrate obtained following the precipitation of blood proteins by tri-chloroacetic acid has then been used by other workers for the extraction of procaine. As a rule they have made the filtrate alkaline (pH 9 or greater) and then extracted it with chloroform (11, 15, 17) or ethylene dichloride (4) without considering any loss of procaine in this alkaline medium.

Initial experiments in this study were directed to the measurement of stability of procaine in a medium at a pH about 7. A series of procaine solutions were made up in phosphate buffer with a pH of 7.36, the concentrations of procaine base ranging from 100 mg./L. to 0.78 mg./L. Equal volumes of procaine-base solution and chloroform were shaken together vigorously after which spectro-photometric analyses in the DK Spectrophotometer were carried out on the chloroform layers. If the original procaine dilution was greater than 15 mg./L., the chloroform layer was then diluted with chloroform so that the concentration of procaine was 10 mg./L. The heights of the curves were proportional to the procaine concentrations and were so similar to curves obtained with aqueous solutions of procaine at the same pH values that loss of procaine could be considered to be negligible.

The recovery of PABA was determined in a like manner. After a phosphate buffer solution (pH 7.36) containing 3.3 μg. PABA was extracted with chloro-form on a roller extraction apparatus for one hour, only 0.8 per cent was

recovered, a quantity much less than that reported (10) for a chloroform-isopropanol solvent (10 per cent PABA at pH 7.0 and 5 per cent at pH 7.5). Thus chloroform alone, appears to be a favourable extracting solvent for procaine at a pH of 7.36, removing only a negligible amount of PABA.

In the present procedure, chloroform removes unknown substances from blood together with procaine, these pass into a N/20 hydrochloric acid extract of the chloroform and may be removed by shaking the aqueous extract with ether. A control extraction made on an N/20 hydrochloric acid solution containing 3.3 µg procaine showed that no measurable quantity of procaine was removed by the ether

METHODS DEMONSTRATING THE PRESENCE OF PROCAINE IN SOLUTION

The quantity of procaine in solution may be estimated in two ways, either by measuring its disappearance when broken down by an enzyme or by alkali per unit time, or by estimating the amount actually present in the solution following the formation of an azo dye from it. The methods recommended for this purpose have been tried and are reported below.

Esterase Method

To determine the efficacy of the esterase method, an esterase solution is made up by adding 0.25 ml. Cholase (trade name for plasma cholinesterase) to 75 ml. distilled water. This solution is equal in cholinesterase activity to that of undiluted human plasma. Following the addition of such a solution to a procaine solution, the rate of breakdown of procaine could be measured in the DK Spectrophotometer (290 mµ) and the loss of procaine estimated for the first four minutes at thirty-second intervals and at the end of two, four, six, and twenty-four hours It was found that in ten solutions thus analysed the reading at the 24-hour period indicated a higher concentration of procaine than was present at the end of the two-hour period; it was then learned by spectrophotometric examination (DK) that this substance was not procaine It was therefore assumed that in the 24-hour period some other material had accumulated and to determine the loss in procaine the final readings should be taken at the end of two hours.

Diazotization

This method appears to be the most popular among investigators. It is valuable for determining diazotizable primary aryl amines with a free amino group or a blocked amino group which can be detached by hydrolysis. The reaction occurring is indicated in Figure 1. Procaine is diazotized with sodium nitrite in acid solution, the excess n trite being removed with ammonium sulfamate to prevent formation of nitroso compounds which would inhibit coupling. Following this, coupling components (19) can be added, that chosen in this experiment being N-(1-naphthyl)-ethylenediamine dihydrochloride which forms an azo dye (purple) upon coupling; this coupling proceeds most rapidly in the pH range of 1 or 2 in the presence of hydrochloric acid. The peak of absorption for N-(1-naphthyl)-ethylenediamine hydrochloride is reported to be 545–550 mµ (11, 16).
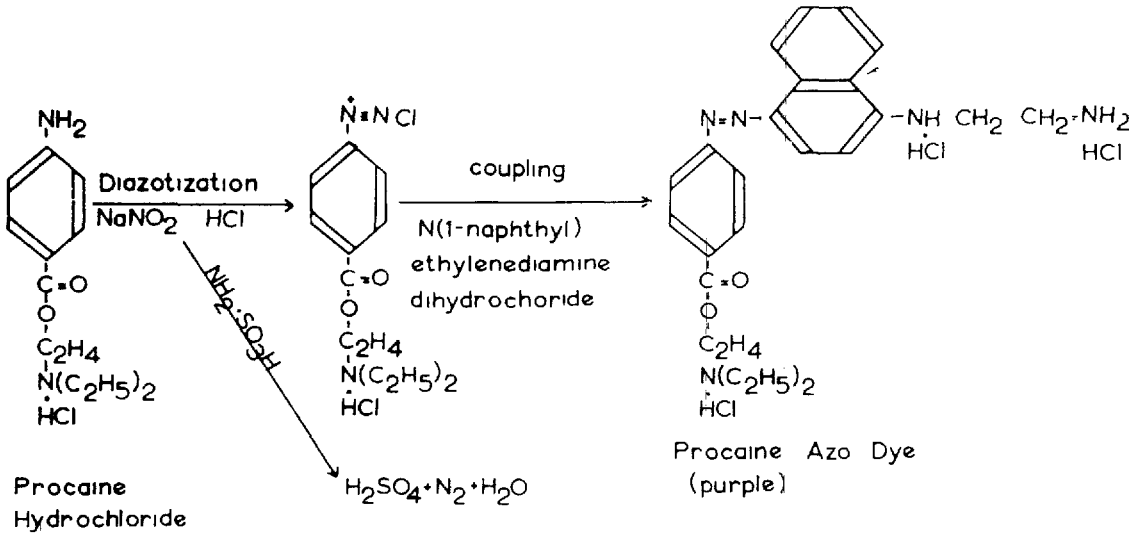
FIGURE 1 Diazo reaction for procaine

A series of test solutions were made up containing 10 μg./ml to 1 156 μg /ml. of procaine hydrochloride. In the analysis, one drop of 1 N hydrochloric acid, the sodium nitrite, ammonium sulfamate, and N-(1-naphthyl)-ethylenediamine dihydrochloride were added in order, to 3 ml. of procaine hydrochloride solutions; the amounts and concentrations of the coupling reagents are recorded in the modified procedure described later. The blank consisted of 3 ml water plus one drop of 1 N hydrochloric acid and the other reagents. The optical densities were measured in the DU Spectrophotometer and the readings thus obtained were plotted against the concentration of procaine hydrochloride (Fig. 2). A straight line was obtained passing through the origin, indicating a direct relation between optical density and concentration of procaine solution This method was the simplest for measuring concentrations of procaine
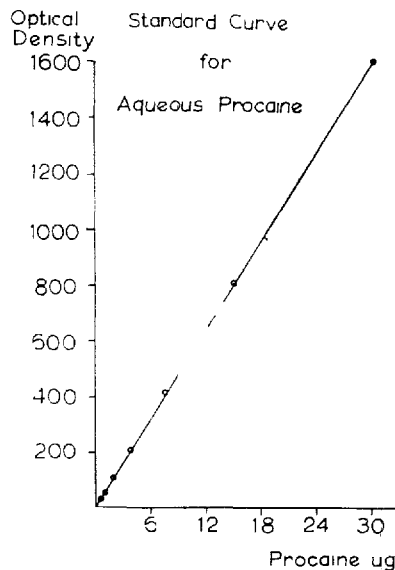

FIGURE 2 The direct relationship between procaine concentration and optical density.

Since samples of blood containing procaine were withdrawn from patients receiving pentobarbital and meperidine, trials were made to ascertain whether these drugs singly or combined would interfere with the method outlined later. Since no interference was found and since sulfonamides were not administered to these patients, it was assumed that this method was satisfactory for the analysis of procaine in blood of persons to whom the drug had been administered.

QUANTITATIVE DETERMINATION OF PROCAINE IN HUMAN BLOOD

Reagents

1 A solution of sodium fluoride-potassium oxalate containing 8.4 gm. sodium fluoride and 3.68 gm. potassium oxalate dissolved in 250 ml. distilled water

2. Chloroform and ether solvents

3. Hydrochloric acid N/20

4 A 0.1 per cent solution of sodium nitrite

5 A 0.5 per cent solution of ammonium sulfamate

6. A 0.1 per cent solution of N-(1-naphthyl)-ethylenediamine hydrochloride which should be kept in the dark

Procedure

To 5 ml. of sodium fluoride–potassium oxalate solution, 3 ml. of blood are added to give a final concentration of M/2 sodium fluoride and M/20 potassium oxalate (pH 7.9). Chloroform 50 ml. is then added to this mixture and the solution is rolled in a suitable bottle on a roller extraction apparatus for one hour, at the end of which time 40 ml. of chloroform are removed. A further extraction employing 40 ml. of chloroform is carried out, rolling for fifteen minutes, after which time 40 ml. of chloroform are again removed. The combined chloroform extracts of 80 ml. are added to 4 ml. of N/20 hydrochloric acid in a separatory funnel. After adequate agitation of the contents, the lower chloroform layer is drawn off and discarded. Solvent ether 3 ml. is then shaken with the acid solution in the separatory funnel to remove any interfering materials which might be present. The lower acid layer is drawn off and freed from any remaining chloroform or ether by evaporation before a fan until the odour of the solvents is no longer present. A blank which is to be used for the zero setting contains 3 ml. of N/20 hydrochloric acid. The following reagents are then added in order to 3 ml. portions of the acid or blank: 0.15 ml. of sodium nitrite solution and allow to stand for five minutes; 0.15 ml. of ammonium sulfamate solution and allow to stand for three minutes; 0.15 ml. of N-(1-naphthyl)-ethylenediamine dihydrochloride solution and allow to stand for twenty minutes for full colour development. The optical densities are measured in the DU Spectrophotometer at 550 mμ wave length and 0.02 mm. slit width, using a tungsten lamp as a source of light.

PREPARATION OF A STANDARD CURVE FOR PROCAINE EXTRACTED FROM WHOLE BLOOD

A graph was prepared with a standard line from which readings could be transposed to quantities of procaine (Fig. 3). For this purpose a procaine solu-

tion containing 20 mg./L. was prepared so that accurate concentrations could be made readily by suitable dilutions. Each of three blood samples 1.5, 3.0, and 6.0 ml. was diluted with procaine so that in each blood sample there were 20, 10, and 5 μg. procaine respectively; for background determinations blood samples were diluted with 1 ml. of water. Percentage of recovery by this method was estimated as follows. The curve for the recovery of procaine from blood was plotted as is shown in Figure 3 (b, c). On the same day a curve was made using the same chemical procedure as outlined on 20, 10, and 5 μg. procaine in N/20 hydrochloric acid. The curve for these results is also shown in Figure 3 (a).
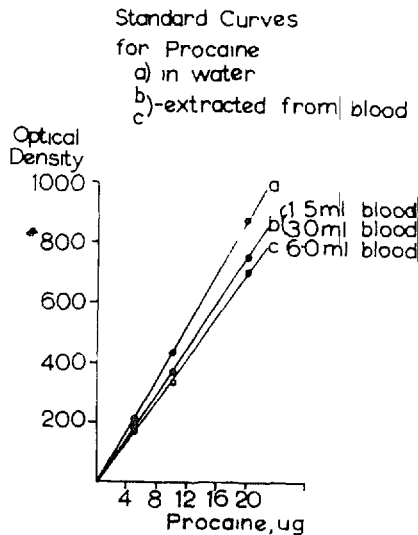

FIGURE 3 The recovery of procaine from water and human whole blood

Using the two curves (a and b or c) the recovery was calculated as follows A point on the curve obtained with the blood samples (1 5 and 3.0 ml.) corresponding to 10 μg. procaine was extrapolated towards the curve of procaine in hydrochloric acid solution parallel to the abscissa. From the point of intersection a line was drawn down perpendicular to the abscissa where the amount of procaine was read to be 8.6 μg. However, since only 40 of the 50 ml. of chloroform were drawn off for the first extraction, the amount of procaine of the original 10 μg. removed at this stage would be 40/50 × 10 = 8 μg., leaving 2 μg. for the second extraction with chloroform; the amount of procaine removed from the second extraction would be 40/50 × 2 = 1.6 μg., Therefore, the total amount of procaine expected from the two extractions with chloroform without any loss should be 8 + 1.6 = 9.6 μg. Thus, the 8.6 μg. above obtained from the graph corresponds to 90 per cent of the expected value (9.6 μg.), indicating that 10 per cent loss occurs during the extraction procedure

While it is evident that the percentage recovery from 6 ml. blood is comparable to that from 1.5 ml. blood, it was found that the second extraction with chloroform was more difficult since emulsification occurred. It was, therefore, decided that blood samples from patients would be in the order of 3 ml. and for greater accuracy the blood would be measured by weight rather than by volume.

METHOD OF WITHDRAWING BLOOD SAMPLES FROM THE ARM VEIN OF A PATIENT

It was recognized that certain errors were bound to occur in the estimation of the concentration of procaine in samples of blood drawn from patients into whom procaine had been injected, because the esterase in the blood would be destroying the procaine during the interval of drawing the sample and transferring it to the sodium fluoride–potassium oxalate solution where the enzyme would be destroyed

The procedure adopted was one in which two syringes connected with suitable stop-cocks were joined to a catheter remaining in the vein during the sampling time (Fig 4) One syringe was filled with saline in order to flush out the catheter, the other was employed to withdraw a blood sample rapidly and to transfer it to the sodium fluoride–potassium oxalate solution in the minimum amount of
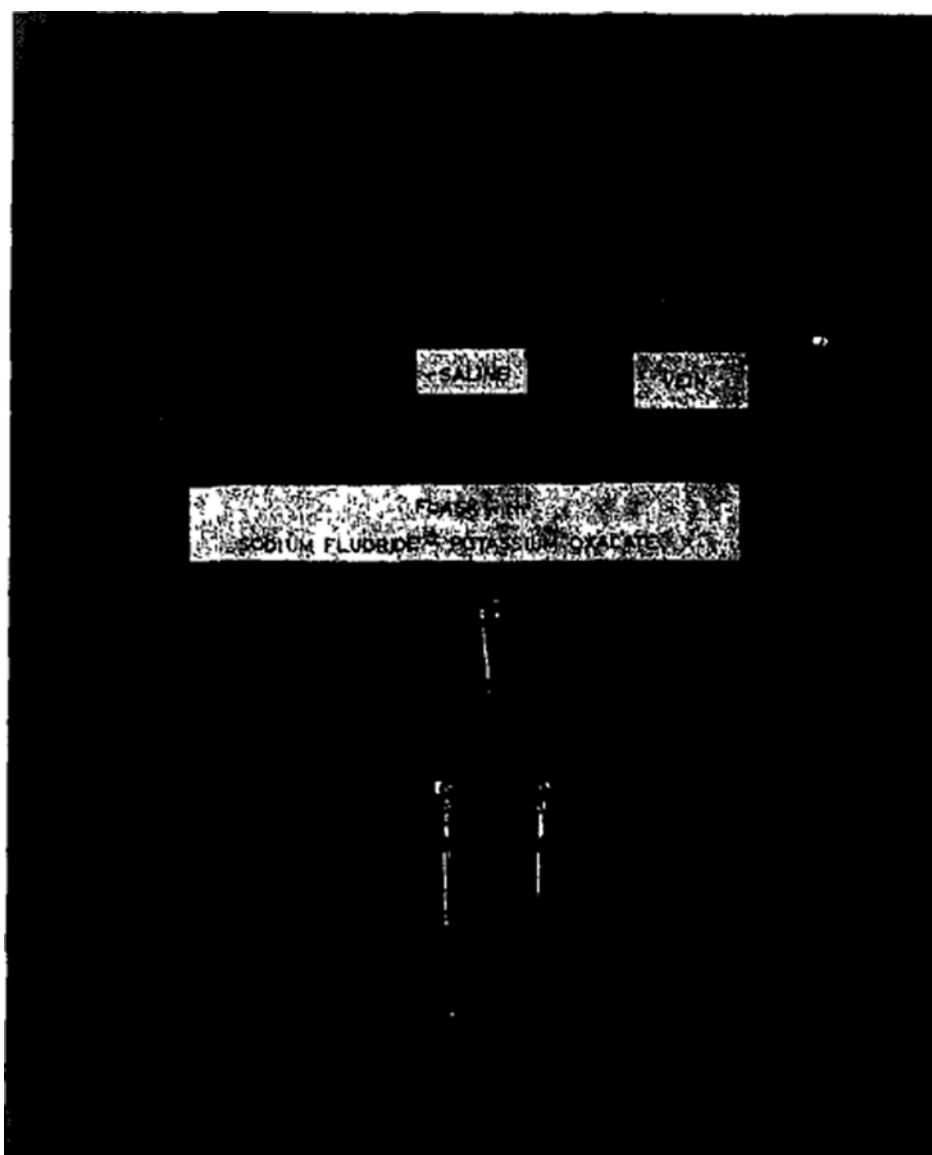

FIGURE 4 Apparatus used for withdrawing blood samples from the arm vein of a patient

time. Time was recorded on the stop-watch from the moment infiltration of procaine (2 per cent solution) was begun, about two minutes were required to complete the subcutaneous infiltration and during this interval the greater part of procaine hydrochloride was injected, varying from 1.7 to 2.5 gm Subsequently small amounts were infiltrated and on occasion the total amount injected rose to 3.7 gm The blood samples were timed from the beginning of infiltration to the time when one-half of the blood in the syringe had been transferred to the sodium fluoride–potassium oxalate, the time interval between samples was approximately five minutes. A control sample of blood for analysis was drawn from each patient prior to the injection of procaine

These investigations were made on male patients undergoing hernia repair, ranging in age from 25 to 50 years, weighing from 148 to 190 lb , and with heights of 5 ft. 6 in., to 6 ft.

RESULTS

The recovery of procaine in these studies is tabulated in Table II and some of the results are presented graphically in Figure 5. These curves were obtained by

TABLE II

CONCENTRATION OF PROCAINE IN WHOLE BLOOD OF SURGICAL PATIENTS AFTER INFILTRATION ANAESTHESIA

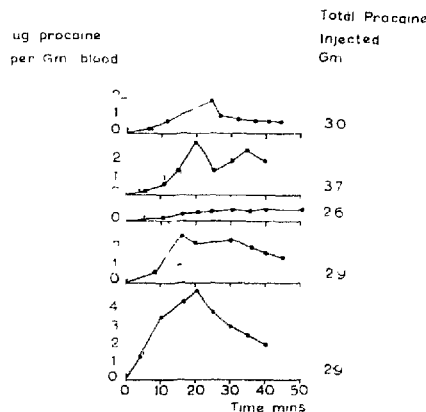| Age (yrs)	Weight (lb)	Height (ft.)	(in)	Total amount injected (gm)	Peak concentration in blood (μg /gm blood)
35	149	5	10 5	3 7	3 0
25	178	6	0	2 8	2 1
35	173	5	6	3 0	1 9
41	185	5	10	3 2	2 2
25	190	5	9	2 9	5 1
36	169	5	10	3 0	1 5
48	170	5	8	3 2	3 2
48	148	5	6	2 06	1 5
39	122	5	7	2 9	2 4
50	160	5	7 5	2 6	0 7
26	175	5	11	2 4	0 7

FIGURE 5. The concentration of procaine in the blood of patients during hernia repair under local anaesthesia

plotting the concentration of procaine determined in the blood at various time intervals after the subcutaneous infiltration of procaine It will be noted that a peak concentration was reached approximately 20 minutes (15–25 minutes) after the injection of procaine, the peak concentration varied from 0.7 to 5.0 μg procaine per gram of blood

DISCUSSION

The method described was employed following a critical examination of other methods reported in the literature, which had been used chiefly for the estimation of procaine injected intravenously. It is believed that by the use of the method described we have estimated with a reasonable degree of accuracy the concentrations of procaine in the blood of patients to whom large amounts of procaine have been given by infiltration The variation seen in the peak concentration (0.7 μg. to 5.0 μg. per gram of blood) may be due to a variation in the serum cholinesterase levels in the blood of the different individuals or in the amounts of procaine injected Further investigations could be designed to determine esterase levels and to follow the procaine concentration in the blood after a single dose of procaine.

SUMMARY

A method which has been developed for the satisfactory estimation of procaine in whole blood avoids the loss of procaine which was apparent in methods where blood proteins were precipitated by use of trichloroacetic acid. Approximately 85 per cent of the added procaine was constantly recovered by this method from test samples of blood and the procedure was considered to be satisfactory for determining concentrations of procaine in patients' blood The peak concentration of procaine in blood twenty minutes after the infiltration of procaine (2.06–3.7 gm) varied from 0 7 μg to 5 0 μg. per gram of blood in the patients studied

RÉSUMÉ

Nous avons mis au point une méthode precise pour mesurer, dans le sang total, la quantité de procaine qui s'y trouve à la suite de filtrations locales de procaine au cours de la chirurgie. Nous exposons les progrès de cette méthode et citons les publications de d'autres méthodes. Nous avons recouvré 85% de la procaine ajoutée à des échantillons de sang humain total, ce qui indique bien que cette méthode est satisfaisante.

La concentration maximale de procaine, variant entre 0 7 mg. et 5.0 mg. par gramme de sang humain total, a été atteinte environ vingt minutes après l'infiltration souscutanée de chlorhydrate de procaine (2 06 à 3 7 gm.) chez des malades subissant une cure de hernie.

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