[Chem. Pharm. Bull. 22(11)2599—2606(1974)]

UDC 547.92'822.3.09 : 615.31.015.4

Determination of Pancuronium Bromide and Its Metabolites in Human Urine by Dye-Extraction Method—Relation between the Extractability and Structure of Quaternary Ammonium Ions[1]

KAZUYO TANAKA, MICHIKO HIOKI, and HIDEYO SHINDO

Central Research Laboratories, Sankyo Co., Ltd.[2]

(Received May 7, 1974)

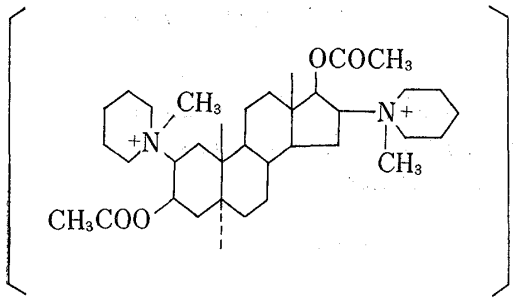
Pancuronium bromide and its deacetylated metabolites were determined in human urine after intravenous administration to five patients by two methods: ion-pair extraction with bromophenol blue for the quaternary nitrogen and hydroxamic acid method for the acetyl group. Succinylcholine which was administered simultaneously was confirmed not to be extracted as the ion-pair complex at all. For the assay of the acetyl group, pancuronium and its metabolites were isolated from the urine as the ion-pair complex followed by column chromatography. The extracts were also subjected to silica gel thin-layer chromatography, wherein pancuronium and its three deacetylated metabolites could be separated while the dye went away to the top of the plate. The results revealed that approximately 20 to 50% of the dose was recovered in the urine during 24 hr period after administration, mostly in the first 3 to 4 hr period, and that pancuronium was excreted mainly as the unchanged form with a slight amount of its mono- and dihydroxy metabolites.

The extractability of quaternary ammonium ions with a wide variety of structure as ion-pair complex with bromophenol blue was examined in order to find any relation to the structure. It was revealed that the extractability is determined mainly by two factors, that is, the lipophylic character of the ammonium ion and the steric effect around the cationic head and the value of the sum of π-constants for four groups plus that of E_s parameters for three smallest groups can provide an approximate measure for predicting the extractability.

Pancuronium bromide, (3α, 17β-dihydroxy-5α-androstan-2β,16β-ylene)-bis-(1-methylpiperidinium)-dibromide diacetate (I),[3] is a new non-depolarizing muscle relaxant containing a steroid nucleus and has been in a wide use clinically.[4] Lübke, *et al.*[5] determined pancuronium bromide in human urine using hydroxamic acid method analysing two acetyl groups and observed only a very low recovery of the drug (about 3% of the dose) in the urine after intravenous administration. More recently, Kersten, *et al.*[6] proposed a more sensitive method of determination using a fluorometric method after ion-pair extraction. The distribution, excretion and metabolism of pancuronium bromide in rats has been reported from this laboratory.[7]

In the present paper, it was aimed to clarify the metabolism of pancuronium bromide in human subjects and pancuronium and its metabolites in human urine were determined using two different methods, that is, analysis of the quaternary nitrogens by dye-extraction method and that of the acetyl groups by hydroxamic acid method. The results indicated

1) This paper was presented at the 15th Local Meeting of Pharmaceutical Soc. Japan, Tokyo, November 1972.
2) Location: *2–58, Hiromachi 1–chome, Shinagawa-ku, Tokyo, 140, Japan.*
3) Pavulon®, Organon Laboratories, Netherland.
4) G. Manani, G. Galzigna, S. Mammano, and A. Gasparetto, *Arzneimittel-Forsch.*, **22**, 1528 (1972); T.M. Speight and G.S. Avery, *Drugs*, **4**, 153 (1972).
5) P. Lübke, G. Wellhelm, and K. Bihler, *Anaesthesist*, **20**, 221 (1971).
6) U.W. Kersten, D.K.F. Meijer, and S. Agoston, *Clin. Chim. Acta*, **44**, 59 (1973).
7) H. Shindo, E. Nakajima, N. Miyakoshi, and E. Shigehara, *Chem. Pharm. Bull.* (Tokyo), **22**, 2502 (1974).



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that 15 to 50% of the intravenous dose was recovered in the urine mostly as the unchanged drug, being dissimilar to those reported by Lübke, *et al.*[5] Furthermore, the relation was investigated between the structure of a wide variety of quaternary ammonium compounds and the extractability of their ion-pair complexes with bromophenol blue.

Material and Metho

Materials——Pancuronium bromide (PC), 3-hydroxypancuronium bromide (3HPC), 17-hydroxypancuronium bromide (17HPC) and 3,17-dihydroxypancuronium bromide (HPC) have been supplied from Organon Laboratories. Methylpropyldiisobutylammonium iodide, dicyclohexyldimethylammonium iodide and tributylpropylammonium iodide were supplied from Prof. T. Fujita of the University of Kyoto. Methylanisotropinium bromide (Valpin®) and its deacylated compound, methyltropinium bromide, were supplied from Endo Laboratories, U.S.A. All other quaternary ammonium compounds tested are the commercial products. Other chemicals used are all reagent grade and used without further purification.

The urine specimens after intravenous administration of pancuronium bromide to patients were kindly supplied from Prof. M. Sato of the Faculty of Medicine, Juntendo University, Tokyo. The urine samples were frozen immediately after collection and stored frozen until the experiments.

Determination of Pancuronium Bromide and Its Metabolites by Ion-Pair Extraction Method——To 20 ml solution containing a known amount of pancuronium bromide was added with 10 ml of 10% sodium carbonate solution and 3 ml of 0.1% bromophenol blue solution. The mixture was then extracted in a 100 ml separating funnel with 40 ml of dichloromethane followed by two successive extraction with 20 ml portions. The extracts were combined to make 100 ml with dichloromethane and the optical density was measured at 602 nm with dichloromethane as reference (Hitachi Model 124 Spectrophotometer). The calibration curve obtained for pancuronium bromide was shown in Fig. 1, as well as that for its deacetylated compound (HPC). As can be seen from the figure, it was confirmed that the dye-extraction method can determine both pancuronium and its all possible deacetylated metabolites by a single calibration curve.

In order to examine a possible interference by succinylcholine which has been usually administered simultaneously with pancuronium bromide, 5 μg/ml succinylcholine was added to the standard solutions of pancuronium bromide. The results, as shown in Fig. 2, indicated that it gives the same calibration curve

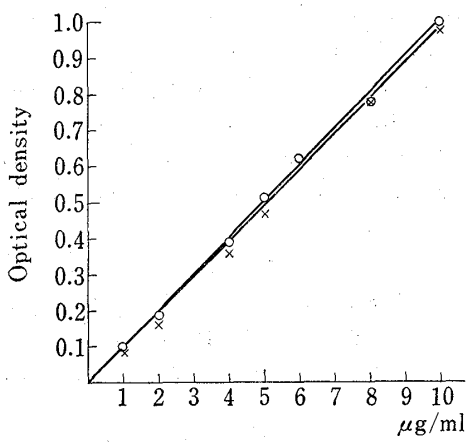

Fig. 1. Standard Curves for Pancuronium Bromide (—○—) and 3,17-Dihydroxypancuronium Bromide (—×—) added to Human Urine

The optical density was measured at 602 nm in dichloromethane extracts (100 ml) from 5 ml urine added with different amount of the compound, as ion-pair complex with bromophenol blue.

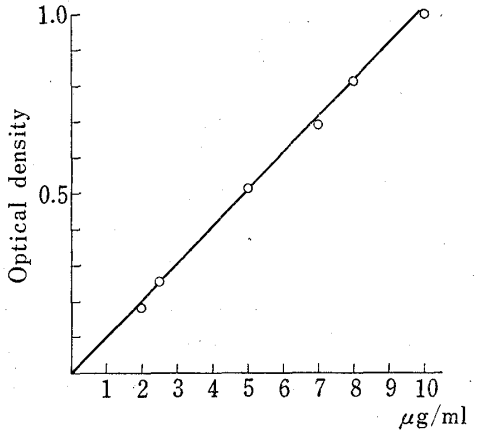

Fig. 2. Standard Curve for Pancuronium Bromide added to Human Urine under Presence of Succinylcholine

The optical density was measured at 602 nm in dichloromethane extracts (100 ml) from 5 ml urine added with different amount of pancuronium bromide and a constant amount (25 μg) of succinylcholine.

as that for only pancuronium bromide (Fig. 1). It was thus confirmed that succinylcholine does not give any extractable ion-pair with bromophenol blue, thus providing a method for determining pancuronium and its metabolites separately from succinylcholine.

The human urine (30 ml) added with a different amount of pancuronium bromide gave the same calibration curve, indicating that there is no interference from urinary background. When it was difficult to separate the dichloromethane layer because of bubbling, the mixture was centrifuged at 2500 rpm for 10 min. The sensitivity of this method was 1 μg/ml final solution, thus 3 μg pancuronium bromide/ml urine.

Determination of Pancuronium Bromide by Hydroxamic Acid Method——One ml aqueous solution containing 0.1, 0.2, 0.5 and 1.0 mg of pancuronium bromide was added with 2.0 ml of 2 M hydroxylamine solution in 2.5 N sodium hydroxide and the mixture was warmed at 40° for 2 hr.[8] After being cooled, the pH was adjusted to about 1.5 by adding 1 ml of 2 N hydrochloric acid and the solution was then colored to red with addition of 1 ml of 0.37 M ferric chloride. The optical density at 500 nm was measured after readjusting the pH to 1.5±0.2 and allowing to stand for 10 min. The calibration curve obtained was represented in Fig. 3, in comparison with that for acetylcholine. The results indicated that the optical density for one acetyl group is the same for pancuronium and acetylcholine. Succinylcholine gave also a comparable curve.

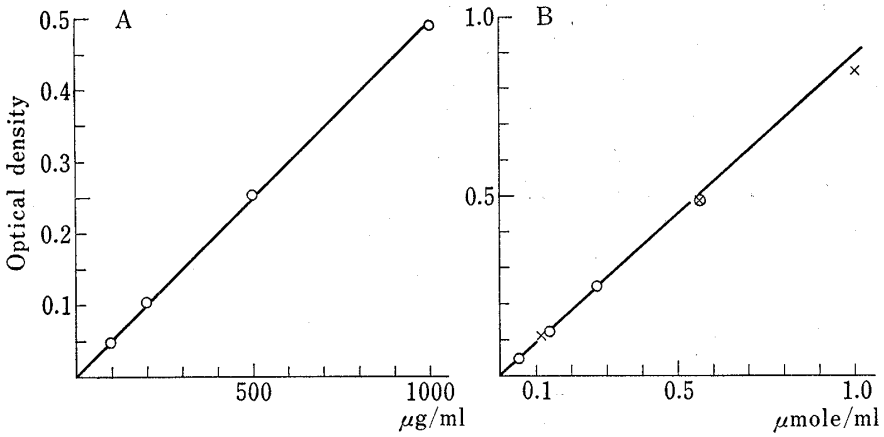

Fig. 3. Standard Curves for Pancuronium Bromide determined by Hydroxamic Acid Method

The optical density was measured at 500 nm in the reaction mixture as described in the text from 1 ml aqueous solution of different amount of pancuronium bromide. In Fig. 3-B, the optical density was plotted against μmole equivalent of acetyl group in comparison with that of acetylcholine (—×—).

Since succinylcholine was contained in most of the urine sample collected from patients, analysis of pancuronium in the urine by hydroxamic acid method was achieved after ion-pair extraction of pancuronium and its metabolites from the sample. The dichloromethane extracts obtained as described in the preceeding section was concentrated to dryness at 20° and the residue was dissolved in 50% ethanol. The solution was then charged on DEAE-cellulose column (12 cm length) and pancuronium and its metabolites could be eluted with 30 ml of 50% ethanol separately from the dye, bromophenol blue, which remained in the column. The eluate was then analyzed for the acetyl groups as described above.

Thin-Layer Chromatographic Separation of Urinary Metabolites——Pancuronium and its metabolites were extracted from the urine as the ion-pair complexes with bromophenol blue in the same way as described. After the dichloromethane extracts were evaporated to dryness under a reduced pressure, the residue was dissolved in a small amount of ethanol and subjected to thin-layer chromatography. Silica gel plate (Merck F_{254}, 0.25 mm thickness) was employed and developed in an open system for 16 hr with the solvent system: the upper phase of *n*-butanol: pyridine: acetic acid: 20% ammonium chloride (60: 40: 12: 48). Authentic pancuronium bromide and three deacetylated compounds were also spotted beside on the same plate as the references. The bromophenol blue complexes dissociated immediately on the development and the dye could be easily separated away to the top of the plate. The spots were visualized by spraying with Dragendorff reagent and the chromatogram was scanned with microdensitometer (Sakura PDM-5). The relative amounts of pancuronium and its metabolites were evaluated by measuring the relative peak areas on the recorded chart.

8) The method described by Lübke, *et al.* (ref. 5) did not give any reproducible result, wherein the mixture with alkaline hydroxylamine was heated at 70° for 75 min and the pH was adjusted to 1.8±0.1.

Result and Discussion

Urinary Excretion and Metabolites of Pancuronium Bromide in Human Subjects

Pancuronium and its metabolites were determined by ion-pair extraction method with bromophenol blue as a counter anion in eight urine specimens collected from five patients (A—E), as shown in Table I. The intravenous dose of pancuronium bromide varying the total amount between 6 and 14 mg was given as a single dose (A and B) or two (C and D) or four (E) successive doses. The urine was collected by cannulation for 24 hr period after the administration or for the first 3 or 4 hr period and until 24 hr afterward. In the four cases, succinylcholine of 20 to 80 mg has been administered simultaneously. As shown in Table I, it was found that 12.4 to 51.9% (average, 36.0%) of the dose was recovered in the urine during 24 hr period after the single or the first administration. It was also noted that most of the excretion appears to occur during the first 3 to 4 hr period, as can be seen from the cases C and D. In patient E, wherein 4 mg was given 15 min after the first 4 mg dose and two successive 2 mg doses 60 and 150 min after the second dose, an appreciable amount was found to be excreted in the period between 3 and 24 hr after the first administration. In all the control urine collected before the administration, no absorption could be detected.

For four samples, the hydroxamic acid method was applied to determine the acetyl groups after extraction of pancuronium and its metabolites as ion-pair complexes with bromophenol blue. The results, as was included in the table, gave the urinary recoveries of pancuronium bromide fairly comparable to those obtained by the ion-pair extraction method, indicating that the most of the urinary metabolites from pancuronium retains its acetyl groups.

Table I. Urinary Excretion of Pancuronium Bromide and Its Metabolites after Intravenous Administration to Five Patients

Patient	Age	Sex	Dose (mg)		Collection of Urine (hr)	Urinary recovery (% to dose)[a]	
			Pancuronium bromide	Succinyl-choline		Dye-extraction method	Hydroxamic acid method
A	52	female	14	20	0—24	20.2	21.2
B	58	male	8	20	0—24	48.1	—[b]
C	18	male	6[c]	40	0— 4	46.7	53.8
					4—24	0.7	—
D	50	male	12[c]	80	0— 3	11.9	12.5
					3—24	0.5	—
E	30	male	12[d]	0	0— 3	31.8	21.5
					3—24	20.1	—

a) The values represent the mean from two or three determinations.
b) not determined
c) Divided doses of 4 and 2 mg and 8 and 4 mg with 30 and 90 min interval in C and D, respectively.
d) Divided dose of 4, 4, 2 and 2 mg with 15, 60 and 90 min intervals.

In order to confirm the above result, the thin-layer chromatographic separation was achieved for the two samples (from pateint D and E). As described in the experimental section, when the ion-pair extracts from the urine were developed on Silica gel plate the dye was separated apart to the top of the plate and the spots of pancuronium and its deacetylated metabolites were detected by Dragendorff reagent and identified from comparison with authentic samples. As shown in Fig. 4, the results revealed a strong spot at the position corresponding to pancuronium, a faint spot corresponding to monohydroxy-, probably 3-hydroxy-, pancuronium and, in the sample from D, another faint spot corresponding to dihydroxy-pancuronium. The densitometry of the chromatograms revealed that the most of the urinary metabolites was unchanged pancuronium (about 75 and 89%) with a much smaller amount of monoacetyl (about 11%) and, in the sample from D, dihydroxy metabolites (about 14%).

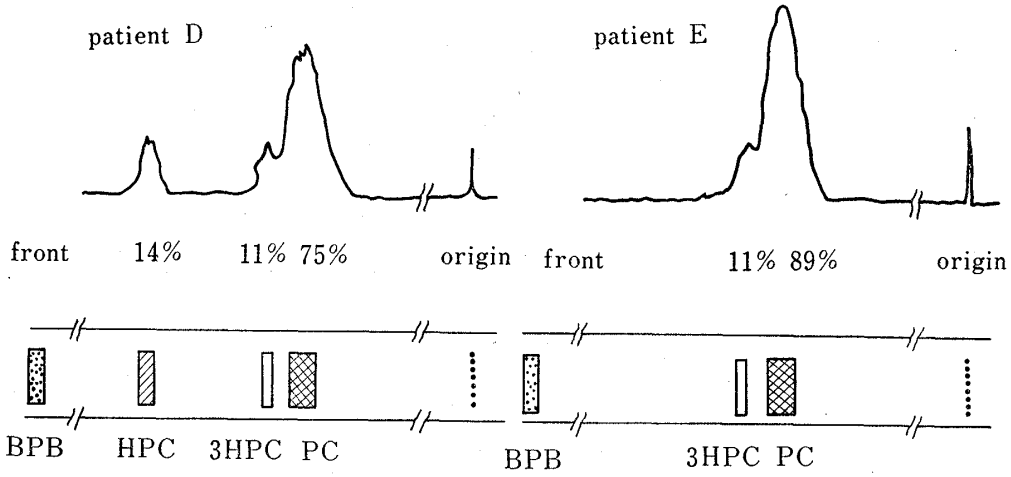

Fig. 4. Thin-Layer Chromatographic Separation and Densitometry of Urinary Metabolites after Intravenous Administration of Pancuronium Bromide to Patients

The urinary metabolites were extracted with dichloromethane as the ion-pair with bromophenol blue and the extracts separated on silica-gel plate using solvent system: upper phase of *n*-butanol: pyridine: acetic acid: 20% ammonium chloride (60:40:12:48). The spots were visualized with Dragendorff reagent and the relative amounts were estimated from the densitometry followed by planimetry.

These results are in contrast to those obtained by Lübke, *et al.*,[5] wherein the hydroxamic acid method was applied and only about 3% of the dose has been recovered in the urine during 24 hr period after the intravenous administration to patients, and a possibility that pancuronium bromide is metabolized extensively in human subjects has been deduced. It has been reported that in rats[7] about 75% of the intraperitoneal dose was recovered in the urine during 24 hr period, while in dogs[9] 38 to 67% of the dose (average from four, 46.5%) during the same period, mostly as unchanged pancuronium in both cases. It has been also reported in rats[7] and dogs[9] that pancuronium is accumulated in the liver and a high concentration continues for a long period particularly in dogs, approximately 8 and 35% of the dose being retained in the liver of rat and dog, respectively, 24 hr after administration. A possibility of a high participation of biliary excretion of pancuronium in human subjects is improbable, since it has been reported[10] that the biliary excretion of pancuronium is insignificant in rat isolated perfused liver. Therefore, it might be most probable in human subjects, just as in the case of dogs, that after an initial rapid elimination of pancuronium through the urinary route a large portion of the drug is accumulated in the liver, which is then very gradually eliminated through the urinary route with a very long half-life. Even in the case of rats, pancuronium-^{14}C in the liver became negligible after more than 30 days.[7]

Relation between Extractability as Ion-Pair with Bromophenol Blue and Structure of Quaternary Ammonium Compound

As described above, both pancuronium bromide and its deacetylated metabolites could be extracted by dichloromethane quantitatively as ion-pair complexes with bromophenol blue, while succinylcholine and, probably, its metabolites such as succinyl-monocholine could not be extracted at all. It is of particular interest and importance in the analytical application of this method to clarify what structural factors play roles in determining the extractability of quaternary ammonium compounds. Although the dye-extraction method has been well known for long years,[11] there are only a few investigations as to the relation between the

9) F. Van der Veen, Unpublished Results from Organon Laboratories, Oss, The Netherland (1971).
10) D.K.F. Meijer and J.G. Weitering, *Europ. J. Pharmacol.*, **10**, 283 (1970).
11) M.E. Auerbach, *Ind. Eng. Chem., Anal. Ed.*, **15**, 492 (1943).

extractability and the structure of quaternary ammonium compounds. It has been reported by Mitchell, *et al.*[12] that only compounds of the general structure which includes at least one chain of four carbons or longer, or benzyl group can be determined by the general extraction method and more recently by Schill[13] that the extraction constant of the bromothymol blue complex increases with increasing number of carbon atoms in the compound, but more generalized rule has not yet been defined.

In order to see at first the stoichiometric relation in the formation of ion-pair complex, the optical density was measured after extraction of pancuronium and cetyltrimethylammonium in a varying concentration between 0.5 and 4.0×10^{-6} moles/ml under presence of a constant amount of the dye, 1×10^{-6} moles/ml. As shown in Fig. 5, the results revealed that the maximum optical density was attained when the molar ratio of quaternary ammonium to dye was 1: 1 and 2: 1 for pancuronium and cetyltrimethylammonium, respectively, indicating that one bromophenol blue molecule forms ion-pair with two quaternary nitrogens.

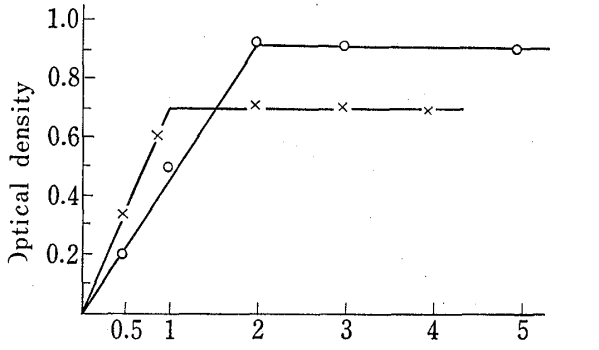

Fig. 5. Stoichiometry in the Ion-Pair Formation of Pancuronium (—×—) and Cetyltrimethylammonium (—○—) with Bromophenol Blue under Alkaline Condition

The optical density was measured at 602 nm in dichloromethane extracts from alkaline solution of different concentration of the compounds under a presence of constant amount (1×10^{-6} moles/ml) of bromophenol blue.

In Table II, the extractability was compared for quaternary ammonium compounds with different structure or chain length in terms of the apparent molar extinction coefficient at 602 nm after the standard method of three times of extraction. The compounds were listed according to the increase in the carbon numbers of all alkyl and aryl groups in a molecule, calculated per one quaternary nitrogen. As a structural parameter, the sum of π-constants of the four groups attached to the nitrogen, which have been derived by Hansch, *et al.*[14] from partition coefficient between *n*-octanol and water, was taken as the measure of lipophilic character of the molecule. The values were calculated by the additive nature of π and the following values were used: 0.50 for CH_3 and CH_2, 2.69 for $C_6H_5CH_2$, —1.16 for OH and —0.27 for $OCOCH_3$. For chain branching, —0.13 and —0.22 was subtracted for secondary and tertiary groupings, respectively, from the value for the normal chain.

As can be seen from Table II, the results revealed that the compounds pocessing its carbon number lower than 10, such as succinyl choline and tetraethylammonium, can not be extracted at all, while those pocessing the carbon number between 11 and 13, such as trimethyloctyl- and tetrapropylammonium can be very poorly extracted. On the other hand, the apparent molar extinction coefficient increased with increasing the carbon number over 14, *e.g.* dicyclohexyldimethylammonium giving the value of 0.17, while all the compounds pocessing the carbon number of more than 15 including pancuronium gave the value exceeding 0.3, approaching to the maximum value of about 0.45. It is suggested, therefore, that the extractability depends mainly on the lipophilic character of the parent quaternary ammonium compound. In fact, the extractability increased roughly with increasing the sum of π-constants of the four groups and it might be said that the compound becomes to be extractable when

12) R. Mitchell and B.B. Clark, *Proc. Soc. Exptl. Biol. Med.*, **81**, 105 (1952).
13) G. Schill, *Acta Pharm. Suecica*, **2**, 13 (1965).
14) J. Iwasa, T. Fujita, and C. Hansch, *J. Med. Chem.*, **8**, 150 (1965); C. Hansch, A.R. Steward, and J. Iwasa, *ibid.*, **8**, 868 (1965); C. Hansch and S.M. Anderson, *J. Org. Chem.*, **32**, 2583 (1967).

Table II. Structure and Ion-Pair Extractability with Bromophenol Blue in Various Quaternary Ammonium Ions

Compound	Carbon number[a]	MEC $\times 10^4$[b]	Sum of π-constants[c]
Choline chloride	5	0	1.34
Acetylcholine chloride	6	0	2.23
Succinylcholine chloride	6	0	2.23
Tetraethylammonium iodide	8	0	4.00
Methyltropinium bromide	9	0	2.95
Trimethylbenzylammonium chloride	10	0	4.19
Tripropylethylammonium iodide	11	0	5.50
Trimethyloctylammonium iodide	11	0.071	5.50
Tetrapropylammonium bromide	12	0.024	6.00
Methylpropylisobutylammonium iodide	12	0.028	5.60
Triethylbenzylammonium chloride	13	0.008	5.69
Dimethyldicyclohexylammonium iodide	14	0.166	6.74
Tributylpropylammonium iodide	15	0.289	7.50
Trimethyldodecylammonium chloride	15	0.476	7.50
3,17-Dihydroxypancuronium bromide	15.5	0.314	5.85
Methylanisotropinium bromide	16	0.387	6.84
Tetrabutylammonium chloride	16	0.422	8.00
Pancuronium bromide	16.5	0.392	6.74
Trimethylcetylammonium chloride	19	0.465	9.50
Benzethonium chloride	27	0.477	9.89

a) The carbon number was expressed as that in all alkyl and aryl groups per one quaternary nitrogen in the molecule. The acetyl group was counted as 1 alkyl carbon.
b) MEC represents the apparent molar extinction coefficient calculated after determination of the optical density at 602 nm by the standard procedure.
c) The values were calculated from π-constants for aliphatic compounds derived from partition coefficient between *n*-octanol and water by Hansch, *et al.* (ref. 14).

the value exceeds the border line of about 5.5. It might be thus understood that pancuronium and its deacetylated metabolites could be extracted well, while succinyl choline could not be extracted at all. It is of interest to note, on the contrary, that anisotropinium can be extracted well, while its deacylated product, methyltropinium, can not be extracted at all, providing a method of determining the former separately.

Table III. Relation between Structure and Ion-Pair Extractability with Bromophenol Blue in Some Tetraalkylammonium Ions

Structure of ion	Carbon number	Extraction %[a]	Sum of π-constants	Sum of E_s parameters	$\sum\pi+\sum E_s$
$(C_2H_5)_4N^+$	8	0	4.00	−0.21	3.79
$(n\text{-}C_3H_7)_3(C_2H_5)N^+$	11	0	5.50	−0.79	4.71
$(CH_3)_3(n\text{-}C_8H_{17})N^+$	11	9.2	5.50	0	5.50
$(\text{iso-}C_4H_9)_2(n\text{-}C_3H_7)(CH_3)N^+$	12	3.2	5.60	−1.29	4.31
$(n\text{-}C_3H_7)_4N^+$	12	4.9	6.00	−1.08	4.92
$(CH_3)_2(\text{cyclo-}C_6H_{11})_2N^+$	14	11.0	6.74	−0.51	6.23
$(n\text{-}C_4H_9)_3(n\text{-}C_3H_7)N^+$	15	61.0	7.50	−1.14	6.36
$(CH_3)_3(n\text{-}C_{12}H_{25})N^+$	15	84.6	7.50	0	7.50
$(n\text{-}C_4H_9)_4N^+$	16	66.9	8.00	−1.17	6.83
$(CH_3)_3(n\text{-}C_{16}H_{33})N^+$	19	100.0	9.50	0	9.50

a) The extractability was estimated from apparent molar extinction coefficient after a single extraction with dichloromethane and was expressed as the percentage to that of trimethylcetylammonium for which 100% extraction was assumed.
b) calculated from Taft's steric parameters (ref. 15) for the three smallest groups

15) R.W. Taft, "Steric Effects in Organic Chemistry," ed. by M.S. Newman, Wiley, New York, 1956, p. 598.

It might be considered from the theoretical viewpoint, however, that the extractability of the ion-pair complex to organic phase must depend not only on the lipophilic character of the parent quaternary ammonium ion, but also on the strength of the ion-pair bond between the cationic head and the counter anion. In order to evaluate the latter factor, as a measure of the partition coefficient of the ion-pair complex, the percent extractability by a single extraction was compared between some selected series of tetraalkylammonium compounds. The compounds were listed in Table III in the order of increasing the carbon number and the sum of π-constants of four alkyl substituents. It can be seen that the extraction percentage increases roughly in parallel to the sum of π-values, but it is also noted that some discrepancies exist. For example, the percentage is significantly lower in tetrapropyl and tetrabutylammonium as compared to trimethyloctyl and trimethylcetylammonium, respectively, in spite of a larger $\Sigma\pi$ values of the former. This is considered to be due to steric hindrance of alkyl groups around the cationic head in the formation of ion-pair complex, resulting in an weaker bond strength and thus making the ion-pair complex more polar. Thus, as a measure of the steric hindrance, steric parameter, E_s, developed by Taft[15] was taken into consideration and the sum of E_s constants was calculated by assuming that only three groups of less steric hindrance actually participate in the complex formation. If it is further assumed that $\Sigma\pi$ and ΣE_s constants has a contribution of a same degree, it was found that there is a good parallelism between the sum of these two constants and the extraction percentages, as can be seen from the table.

It might be concluded from the present results that the extractability of quaternary ammonium ions as ion-pair complex with bromophenol blue is determined mainly by two factors, that is, the lipophilic character of the ion and the steric effect around the cationic head and that the value of $\Sigma\pi+\Sigma E_s$ can provide an approximate measure for predicting the extractability.

Added in Proof

After completion of this manuscript, we noticed that Agoston, *et al.*[16] reported recently on the urinary excretion of pancuronium bromide in human subjects and the result revealed that approximately 37—44% of the dose was recovered in the urine, in good agreement with our present results.

Acknowledgement The authors express their deep gratitudes to Prof. M. Sato of the Faculty of Medicine, Juntendo University, for his kind supply of the urine specimens, to Prof. T. Tanimura of the University of Tokyo and Prof. S. Tsurufuji of the University of Tohoku for their valuable advices and discussions and to Prof. T. Fujita of the University of Kyoto for supplying some of compounds used and for his valuable discussions. Thanks are also due to Dr. G. Sunagawa, director of this laboratories, and to Dr. K. Tanabe of this laboratories for their kind encouragements.

16) S. Agoston, G.A. Vermeer, U.W. Kersten, and D.K.F. Meijer, *Acta Anaesth. Scand.*, 17, 267 (1973).