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PREPARATION AND BIODISTRIBUTION OF SALICYLIDENE-AMINATES (SCHIFF BASES) LABELED WITH Tc-99m

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SUMMARY

A series of salicylidene-aminoacid Schiff bases was synthesized and labeled with Tc-99m, in neutral pH, using stannous ion as reductant. Labeling yield was greatly affected by concentration-ratios of the reactants, the optimum concentration ratio being 18.5 x 10^{-2}mmol ligand to 0.4 x 10^{-2}mmol $SnCl_2$ per 4 ml formulation.

Various substituents on salicylidene-phenylalanine molecule or the different amino acids used for the imine formation did not significantly influence the yield of the labeling which usually exceeded 90%. Most of the ^{99m}Tc-chelates exhibited high hepatobiliary affinity, when injected intravenously in mice. Blood clearance as well as hepatic extraction rate varied with the chemical structure of the azomethine derivatives. Urinary elimination was negligible except the hydrophilic complexes ^{99m}Tc- salicylidene-isoleucine, ^{99m}Tc- salicylidene-cycloleucine and ^{99m}Tc- salicylidene-alanine which excreted to the urine as well.

* Key Words: Salicylidene-aminates, imines, labeling with Tc-99m, Biodistribution.

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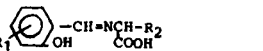
INTRODUCTION

Technetium-99m labeled Schiff bases were first introduced by Baker et al(1,2), as potential hepatobiliary agents to replace ^{131}I-Rose Bengal. The first derivative studied, ^{99m}Tc-pyridoxylidene-glutamate, was formulated by autoclaving pyridoxal, glutamic acid and pertechnetate in alkaline pH. The mixture when injected intravenously, was concentrated rapidly in the liver and excreted via the biliary tree into the intestines. Baker's original work offered a new approach to the development of ^{99m}Tc-hepatobiliary agents, and radiopharmaceuticals in general. Numerous ^{99m}Tc-labeled pyridoxal-aminoacid complexes were then developed(3-5) and evaluated in experimental animals as possible hepatobiliary agents. The chemistry of labeling pyridoxylidene-aminates with Tc-99m was also investigated(6). The proposed reaction mechanism suggested that reduction of pertechnetate and formation of a Schiff base-technetium complex were occuring simultaneously. However, radiochemical impurities such as reduced-colloidal technetium as well as various side-products of transamination reaction which may bind technetium were formed,degrading considerably the imaging quality. The large amounts of radioactivity excreted to the urine were assigned to these impurities.

The preparation of ^{99m}Tc-pyridoxal-aminoacid complexes was further improved via frozen kit formulations using stannous chloride as reducing agent of technetium(7). According to this preparation method, the ^{99m}Tc-pyridoxylidene-aminate complex was considered to be formed via the intermediates tin pyridoxal and tin Schiff-base complexes.

A ^{99m}Tc-salicylidene-aminate complex, excreted mainly via the hepatobiliary system, was later reported(8). It was prepared by mixing salicylaldehyde, phenylalanine, stannous chloride and $^{99m}TcO_4^-$. The complex formed suggested a

TABLE 1. Salicylidene - aminates



Compd	R_1	R_2	Formula	Yield(%)	m.p.°C[a]	Analysis C%	H%	N%	NMR, ppm (DMSO-D_6, *CF_3COOH)
I	H	$-CH_2$⟨⟩	$C_{16}H_{15}NO_3.H_2O$	46	161-163	66.35b (66.88)c	5.38 (5.96)	4.63 (4.87)	3.18 ($CCH_2\Phi$), 4.34 (NCH(COOH)C) 7.2 ($\Phi CH=N$, 2Φ)
II	H	$-CH_2$⟨⟩-F	$C_{16}H_{14}FNO_3$	50	215-217	66.32 (66.89)	4.39 (4.91)	5.19 (4.87)	3.48 ($CCH_2\Phi$), 4.60 (NCH(COOH)C) 7.32 ($\Phi CH=N$, 2Φ)
III	H	$-CH_2$⟨⟩-F	"	35	168-170	66.24 (66.89)	5.27 (4.91)	4.38 (4.87)	3.51 ($CCH_2\Phi$), 4.64 (NCH(COOH)C) 7.41 ($\Phi CH=N$, 2Φ)
IV	H	$-CH_2$⟨⟩-F	"	52	158-160	66.85 (66.89)	5.15 (4.91)	4.37 (4.87)	3.44 ($CCH_2\Phi$), 4.57 (NCH(COOH)C) 7.36 ($\Phi CH=N$, 2Φ)
V	H	$-CH_2$⟨⟩-I	$C_{16}H_{14}INO_3$	26	181-183	49.05 (48.63)	4.02 (3.57)	2.98 (3.54)	3.41 ($CCH_2\Phi$), 4.55 (NCH(COOH)C) 7.5 ($\Phi CH=N$, 2Φ)
VI	H	$-CH_2$⟨⟩-OCH_3	$C_{17}H_{17}NO_4$	25	165-167	67.65 (68.21)	5.13 (5.72)	4.18 (4.68)	3.09 ($CCH_2\Phi$), 3.68 ($\Phi-OCH_3$) 4.26 (NCH(COOH)C), 7.23 ($\Phi CH=N$, 2)
VII	4-OCH_3	$-CH_2$⟨⟩	$C_{17}H_{17}NO_4$	53	167-169	67.74 (68.21)	6.15 (5.72)	5.18 (4.68)	3.75 ($CCH_2\Phi$, $\Phi-OCH_3$, NCH(COOH)C) 6.35 ($\Phi CH=N$), 7.14 (2Φ)
VIII	5-CCH_3	$-CH_2$⟨⟩	$C_{17}H_{17}NO_4.H_2O$	30	274-276	64.60 (64.34)	6.71 (6.03)	5.13 (4.70)	*3.65 ($\Phi-OCH_3$), 4.75 (NCH(COOH)C) 7.51 (2Φ)
IX	5-NO_2	$-CH_2$⟨⟩	$C_{16}H_{14}N_2O_5$	20	127-129	60.85 (61.15)	5.01 (4.48)	8.86 (8.91)	3.39 ($CCH_2\Phi$), 4.67 (NCH(COOH)C) 6.75 ($\Phi CH=N$), 7.22 (2Φ)
X	4-CH	$-CH_2$⟨⟩	$C_{16}H_{15}NO_4$	40	258-260	66.76 (67.35)	5.20 (5.30)	4.75 (4.90)	*3.62 ($CCH_2\Phi$), 4.78 (NCH(COOH)C) 7.50 (2Φ, $\Phi CH=N$)
XI	H	$-CH_2$⟨N⟩	$C_{18}H_{16}N_2O_3.2H_2O$	54	183-185	63.20 (62.78)	6.00 (5.86)	8.80 (8.13)	*3.82 ($-CH_2$), 4.81 (NCH(COOH)C) 7.37 ($-CH=N$, 2Φ)
XII	H	$-CH(CH_3)CH_2\ CH_3$	$C_{13}H_{17}NO_3.2H_2O$	43	273-275	57.70 (57.55)	8.32 (7.82)	5.73 (5.16)	*1.27 ($-CH_3$), 1.73 ($-CCH_2C$) 2.37 (CCHC), 4.51 ($-NCH(COOH)C$) 7.37 (Φ, $CH=N$)
XIII	H	$-CH_2(CH_2)_2CH_3$	$C_{17}H_{17}NO_3$	56	158-160	65.96 (66.36)	6.93 (7.28)	5.52 (5.95)	*1.14 (CH_3), 1.56 ($2-CH_2$), 2.10 (CH_2) 4.33 ($-NCH(COOH)C$) 7.46 (Φ, $CH=N$)
XIV	H	CH_2-CH_2 / CH_2-CH_2	$C_{13}H_{15}NO_3$	26	300-302	66.05 (66.94)	6.00 (6.48)	6.78 (6.00)	*2.25 ($-CH_2$), 7.49 (Φ)
XV	H	$-CH_3$	$C_{10}H_{11}NO_3$	26	274-276	62.69 (62.17)	6.01 (5.74)	7.87 (7.25)	*1.93 ($-CH_3$), 4.56 (NCH(COOH)C) 7.50 (Φ)
XVI	H	$-CH_2CH_2SCH_3$	$C_{12}H_{15}NO_3S.2H_2O$	60	138-140	49.92 (49.81	6.94 (6.63)	5.21 (4.84)	*3.0 (CCH_2C), 4.75 (NCH(COOH)C) 7.6 (Φ)

a.Recrystallized from ethanol, b.Values found, c.Values calculated

^{99m}Tc-labeled Schiff base-type compound.

Complexes of salicylidene-aminates with various metals are already known and determinations of stability constants indicated that these imines are good chelating agents for Cu^{2+}, Fe^{3+}, or Co^{3+}(9-11). All ^{99m}Tc- labeled azomethine derivatives reported so far, were prepared from non-isolated Schiff bases. The imines were formed in situ, using excess of the reactants. Knowledge, however, of the chemical characteristics of the ligands is essential in the development of ^{99m}Tc-radiopharmaceuticals(12).

In this study, we investigated the ability of isolated salicylidene-aminates to form stable complexes with Tc-99m as well as the chemistry of the labeling reaction. Moreover, biodistribution studies of the various ^{99m}Tc-azomethine derivatives were performed in order to examine the in vivo behavior of this class of technetium-complexes.

RESULTS AND DISCUSSION

The structure of salicylidene-aminates prepared in this study, their physical properties as well as elemental analysis and ^{1}HNMR data, are presented in Table 1. Synthesis of crystalline azomethine derivatives was achieved by reacting 0.02 mole of salicylaldehyde with 0.01 mole of the amino acid in ethanol. Preparation of salicylidene-aminates according to the literature stoichiometry(13), 6:1 salicylaldehyde to amino acid, yielded oily recidues, probably due to excess aldehyde.

Schiff bases listed in Table 1 were labeled with Tc-99m by the stannous ion reduction method in neutral pH. Optimization study of the labeling reaction performed in salicylidene-phenylalanine (SPh), indicated that the optimal molar ratio for quantitative ^{99m}Tc- complex formation, is 46:1 ligand to re-

ducing agent (18.4×10^{-2}mmole SPh and 0.4×10^{-2}mmole $SnCl_2$ per 4mL). In these concentrations the labeling method yielded 90% of ^{99m}Tc-SPh while the rest of activity was identified as impurities (free pertechnetate or technetium-colloid). Higher concentrations of the ligand (37.1×10^{-2}mmole) did not alter significantly the labeling yield. Concentrations of imine below 9.2×10^{-2}mmole afforded higher amounts of radiochemical impurities (20% approximately). Low yields of ^{99m}Tc- chelate were also obtained when the quantity of stannous chloride was decreased. Thus, when 0.1×10^{-2}mmole of $SnCl_2$ to 18.5×10^{-2} mmole of SPh/4mL were used, the percentage of ^{99m}Tc-SPh was found as only 45.6% of the total activity. The radiochemical purity was also decreased when the concentrations of the imine and stannous chloride were subdivided to 9.2×10^{-2} and 0.2×10^{-2}mmole respectively. In this case, only 72% of ^{99m}Tc-SPh was recovered.

Radiochemical analysis of the labeled azomethine derivatives revealed that heptavalent technetium was almost completely reduced and quantitatively bound by the various ligands, under the experimental conditions established. In most compounds the percentage of chelated technetium, estimated by both analytical methods, exceeded 90% whereas only negligible amounts of colloidal technetium were detected. ^{99m}Tc salicylidene-aminates moved along with $^{99m}TcO_4^-$ to the solvent front (Rf= 0.7-0.9) when acetone and acetonitrile-water system were used, while colloidal technetium stayed at the origin. In NaCl solutions, lipophilic ^{99m}Tc complexes remained near the origin (Rf= 0.0-0.1). The radiochemical purity of ^{99m}Tc-chelates of compounds I-X was found high, ranging from 90 to 96% by ITLC. In electrophoresis, the complexes migrated from the origin 1-4 cm, the distance varying with the substituent. Substitution in these compounds did not affect greatly the labeling yield. Concerning the derivatives of salicylaldehyde with various amino

acids (compounds XI - XVI) the percentage of complexed technetium varied from 74.5 to 98%. In some cases, lesser amounts of impurities were identified by electrophoresis compared to ITLC analysis. This discrepancy may be attributed to complex instability in the ITLC method used. Radiochemical data of the labeled Schiff bases were moreover confirmed by biological assay in mice 15 and 30 minutes after i.v. administration. Negligible amounts of radioactivity were recovered in stomach and spleen indicating the absence of free pertechnetate and colloidal technetium.

Distribution data in mice of the various ^{99m}Tc-salicylidene-aminates are illustrated in Figures 1-3. Group A presents ^{99m}Tc-complexes substituted in phenylalanine ring, group B salicylaldehyde-substituted phenylalanine derivatives while group C includes ^{99m}Tc-salicylidene-Schiff bases of various amino acids. In figure 1 and 2 the blood clearance and hepatic extraction rate of the complexes of groups A, B and C are shown comparatively. Each value represents the mean per cent injected dose in blood and liver for five animals, plotted against time. ^{99m}Tc-SPh values are included in graph of group B as well as in C (Fig. 2) for comparison. The data demonstate that the various substituents influenced the biodistribution of ^{99m}Tc-labeled imines. Halogenated phenylalanine derivatives (group A , Fig. 1) were cleared faster from the blood than ^{99m}Tc-SPh. Remarkable differences were not observed in the rate of blood clearance between o-F, m-F and p-F derivatives. Hepatic transit of the complexes was only delayed in p-Iodo-derivative,where approximately a 40% of the dose remained in the liver 2 hrs p.i. Substitution in salicylaldehyde moiety (group B , Fig. 1) gave derivatives which cleared from the blood more slowly than simple ^{99m}Tc-SPh. Hepatic extraction rate of these complexes varied according to the

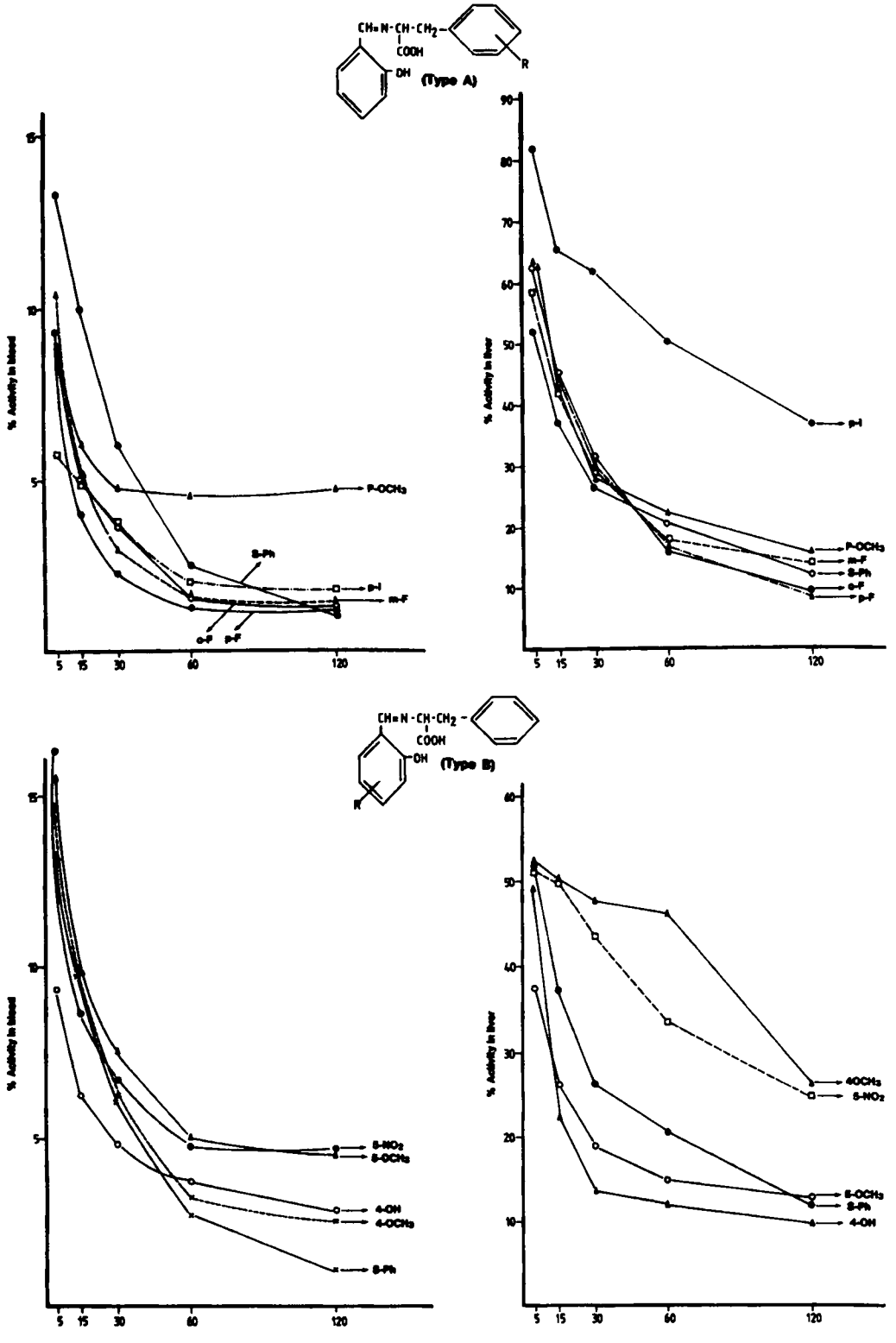


Fig.1: Comparative biodistribution in mice of ^{99m}Tc-salicylidene - phenylalamine derivatives.

substituent. Thus, ^{99m}Tc-5-OCH_3 and 4-OH-SPh were extracted from the hepatocytes to the bile faster than SPh while 5-NO_2 and 4-OCH_2 derivatives were slower. The animal data of ^{99m}Tc-Salicylidene-aminates of group C, presenting in Fig. 2 (where R various amino acids) demonstrate that these compounds have slower blood clearance than complexes I-X. Hepatobiliary transit of these derivatives is generally found to be faster than ^{99m}Tc-SPh, except ^{99m}Tc-salicylidene-trytrophan which cleared slower from the liver at the earlier times p.i. Among these derivatives ^{99m}Tc-salicylidene-methionine has a faster extraction rate from liver to intestines.

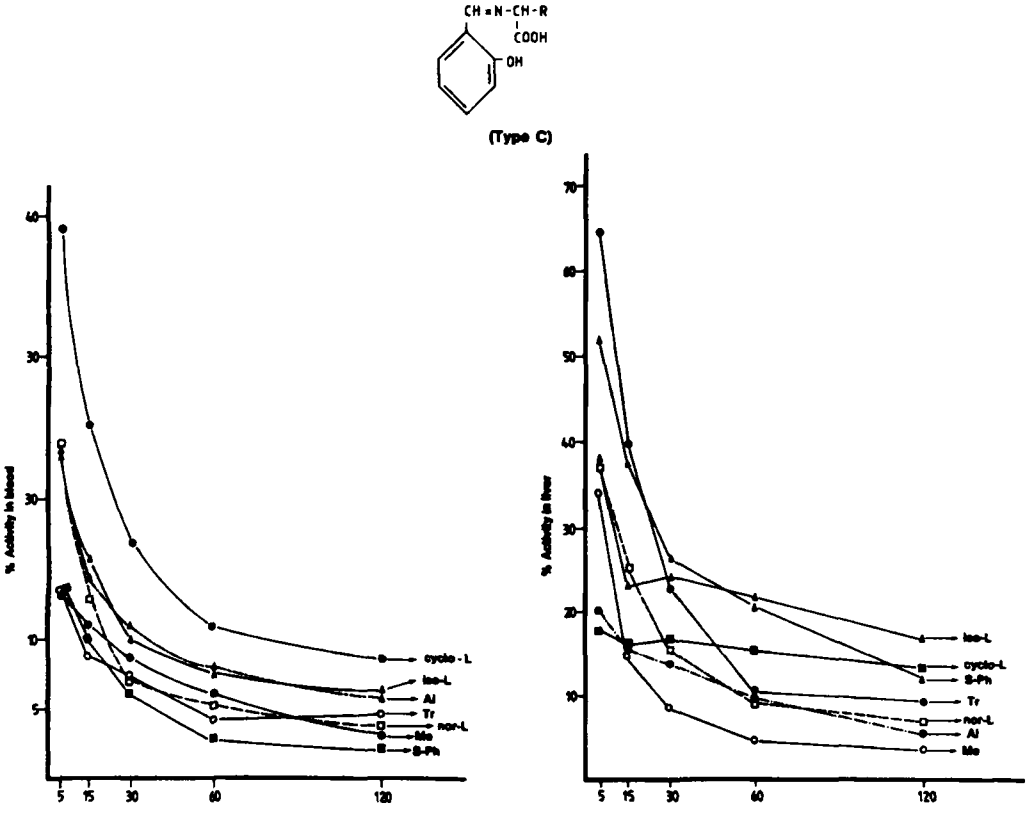


Fig. 2: Comparative biodistribution in mice of ^{99m}Tc-salicylidene aminates.

Figure 3 illustrates the quantitative excretion patterns of the ^{99m}Tc-chelates, 2 hrs after administration. Their octanol-water partition coefficients (P.C.) are reported. Radioactivity recovered into the intestines clearly demonstrate that hepatobiliary system is the main excretory pathway for most of the ^{99m}Tc-salicylidene-aminates studied. Low amounts of activity were found in the urinary bladder except ^{99m}Tc-salicylidene-isoleucine,cycloleucine and ^{99m}Tc-salicylidene alanine

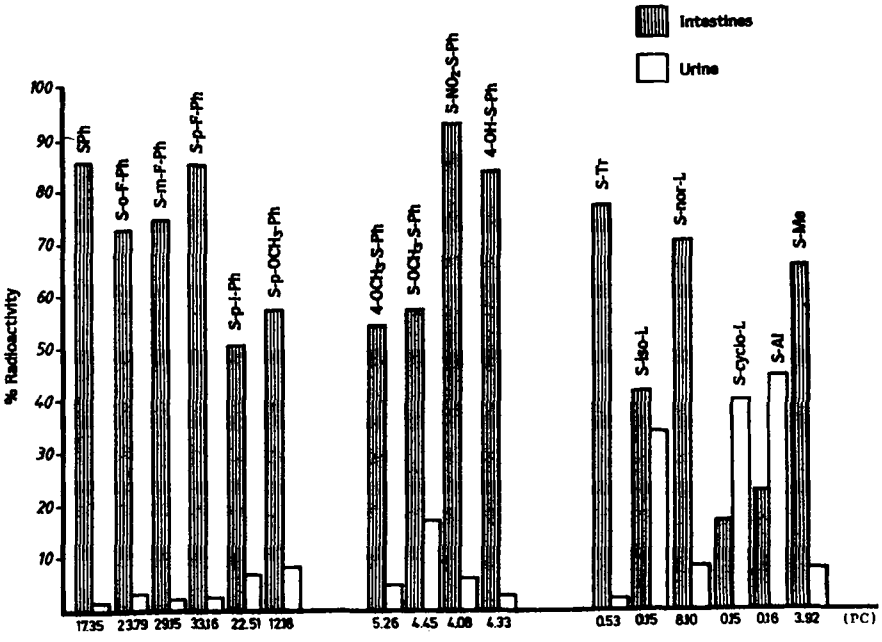


Fig. 3: Elimination pattern of ^{99m}Tc-salicylidene aminates determined in mice at 2 hrs p.i.

which were concentrated to the urine 33.8, 40.1 and 44.7% respectively. Since these complexes are characterized by the lowest P.C. values, increased urinary excretion may be attributed to hydrophilicity. However, partition coefficients of the labeled imines studied cannot be directly correlated to elimination route because several structural parameters may also be involved(14). Thus, ^{99m}Tc salicylidene-tryptophan which showed a comparatively low P.C. value exhibited a highly specific hepatobiliary concentration.

Generally, biodistribution patterns of most of the ^{99m}Tc-complexes studied indicated a high liver affinity and negligible urinary excretion. The elimination via the urinary system is significantly lower compared to ^{99m}Tc-pyridoxylidene-glutamate or similar complexes prepared by the autoclaving method (1) approximately 46% in the urine of mice, 2 hrs p.i.).

^{99m}Tc-SPh was moreover formulated via a freeze-dried Kit, using the optimum concentration ratio 46:1, ligand to stannous ion. Radiochemical purity of the reconstituted Kit, determined by the previously noted analytical methods, proved that the lyophilization process did not influence the in vitro stability of ^{99m}Tc-SPh complex. Biodistribution data of ^{99m}Tc-SPh Kit carried out in mice were similar to those of ^{99m}Tc-SPh instant preparation.

In conclusion, isolated salicylidene-aminates consist of a class of ligands capable of binding almost quantitatively to the reduced Tc-99m. The complexes formed were stable in vitro and in vivo. Various substituents on salicylalaldehyde-Schiff bases did not significantly influence the yield of the labeling reaction which was found to be greatly affected by concentration-ratios of the reactants. Optimum concentration ratio for efficient labeling was found to be 18.5 x 10^{-2} mmole imine to 0.4 x 10^{-2} mmole $SnCl_2$ per 4 mL. Most of the ^{99m}Tc-chelates were selectively excreted via the hepatobiliary system. Blood clearance as well as hepatic extraction rate was influenced by the substitution on salicylidene-phenylalanine molecule or by the use of various amino acids, for the imine formation. Certain compounds such as ^{99m}Tc-SPh, ^{99m}Tc-4-OHSPh, and ^{99m}Tc-STry showed interesting biological characteristics i.e. fast hepatobiliary transit, low urinary excretion and could be considered as possible hepatobiliary radiopharmaceuticals. The latter requires confirmation by fxrther evaluation in higher animal models.

EXPERIMENTAL

Melting points were determined in capillary tubes using a Buchi apparatus and are uncorrected. Elemental analysis were performed by the Microanalytical Laboratory of the National Hellenic Research Foundation. Nuclear magnetic resonance (NMR) spectra were obtained at 80 MHz with a Varian FT-80 A instrument or at 100 MHz on a Varian XL-100 A spectrophotometer. Samples 30-40 mg) were dissolved in DMSO-D_6 or CF_3COOH and the resonances are reported downfield (δ,ppm) from the internal tetramethylsilane standard. All pH measurements were carried-out with a Metrohm-632 digital pHmeter, at room temperature. Radioactivity was counted in a Picker-Isotope Calibrator or in a well-type NaI(Th) γ-counter (ICN-GS 500 model).

Materials and methods. All chemicals and solvents were of analytical grade and were used without further purification. The various o-hydroxy-benzaldehydes and amino-acids were commercialy obtained. Sodium ^{99m}Tc-pertechnetate was eluted from a commercial ^{99m}Tc-generator (CIS). Stannous chloride (grade I) was supplied by Sigma Chem. Co. Instant Thin Layer Chromatography (ITLC) tests were performed on silica-gel impregnated glass fiber strips (Gelman ITLC, type SG) and electrophoresis experiments on Whatman paper No 1 with tris-barbital-sodium barbital electrophoresis buffer (Gelman Chem. Co.).

Synthesis of salicylidene-aminates: A general procedure was used as follows:
To a solution of 2.44 g (0.2mmole)of salicylaldehyde in 300 mL of ethanol, 0.1mmole of the amino acid was added. The mixture was refluxed for 6 hrs, concentrated in vacuo and left at room temperature to give the grude product. Recrystalization of the imines from EtOH afforded yellowish to yellow products in yield 20-60%.

R a d i o l a b e l i n g: The various azomethine derivatives were labeled with Tc-99m by the stannous ion reduction method. Stannous chloride solution was always freshly prepared in order to avoid Sn^{2+} oxidation.

Optimization of the labeling procedure was performed on salycylidene-phenylalanine (SPh). Thus, concentrations of the ligand and $SnCl_2$, ranging from 1.1 to $37.1x10^{-2}$ and $0.1-0.4x10^{-2}$mmole respectively, were mixed with $^{99m}TcO_4$, (30-50 μCi) at the appropriate pH in 4 mL final volume. Aliquots of the various reaction mixtures were analysed by ITLC and electrophoresis and the percentage of complexed, reduced, and unbound technetium were calculated. Biodistribution followed the formulations in order to evaluate the analytical results. Molar ratio 46:1 ligand to reducing agent gave the best labeling yield. Based on these data the following labeling procedure of salicylideneaminates was formulated: The Schiff base ($18.5x10^{-2}$ mmole), was suspended in 2 mL of distilled water and 10% NaOH was added dropwise with vigorous magnetic stirring, until complete dissolution (pH≃11.0). The pH of the mixture was then adjusted to 6.5-7.0 with 1 and 0.1 N HCl, and 0.1 mL of stannous chloride solution (5 mg/mL in 5N HCl) was added with continious stirring. The pH was finally adjusted to 7.4 with 1N and 0.1N NaOH. The mixture was filtered through a Millipore filter (0.22 μm) in an evacuated penicilline vial and 30-50 μCi of Na $^{99m}TcO_4$ solution in the appropriate volume, were added to the vial, in order to obtain final solution volume of 4.0 mL. After agitation of the mixture for 3 minutes at room temperature, the vial was ready for further use.

Labeling of salycilidene-p-iodo-phenylalanine, 4-methoxy-salicylidene-phenylalanine and 5-nitro-salicylidene-phenylalanine was achieved by using $9x10^{-2}$ mmole, due to their high insolubility.

Radiochemical analysis: Percentage of ^{99m}Tc-ligand and radiochemical impurities in various preparations were determined by Instant Thin Layer Chromatography (ITLC) and electrophoresis.

Four chromatographic systems were used for ITLC analytical procedure: dry acetone, acetonitrile-water (3:1 V/V), NaCl 0.9% and NaCl 2%. A drop of each complex solution was charged on the chromatographic strip (12x0.8 cm) and developed at 10 cm. The chromatograms were cut into sections of 1 cm and counted in a well-type γ-counter. The percent activity of each section was calculated and the Rf values from areas with increased radioactivity were recovered as follows: The percentage of unbound technetium (T) was determined in NaCl solutions. The sum of the complexed and unbound technetium (S) as well as of the reduced technetium ($^{99m}TcO_2$) were determined in acetone or acetonitrile-water system. The percentage of complexed technetium was calculated by subtracting T from S.

For electrophoresis, a tris-barbital buffer pH 8.8 and ionic strength 0.06 was used. The electrophorograms were run for 1.5 h at constant voltage 200 V. The strips were dried at room temperature and cut in 1 cm sections. The Rf values and the percentage of ^{99m}Tc-compounds were determined as noted in chromatography procedure.

Lipophilicity measurements: Partition coefficient of the various ^{99m}Tc-complexes were determined in n-octanol-isotonic phosphate buffer (0.05 M, pH 7.0) system.

In a test-tube charged with phosphate buffer and n-octanol (2 mL each) 100 λ of the complex solution was added. After vigorous mixing on a Vortex mixer for 10 min, the mixture was centrifuged (3000 G, 10 min) so as to complete the phase separation. An aliquot (0.2mL) of each phase was pipetted into a counting vial, weighed and counted in a γ-counter. Special care was

taken to prevent the intercontamination between each phase. Partition coefficient (K_D) of each labeled compound was calculated as follows:

$$K_D = \frac{\text{Counts/g octanol}}{\text{Counts/g phosphate buffer}}$$

Biological studies: Male Swiss white mice, 20±2 g, were injected intravenously through the tail vein with 0.2 mL of the ^{99m}Tc-complex solution. In order to collect urine, the animals were put in metabolic cages. Groups of five mice were sacrified with ether-vapors 5 - 30 min., 1 and 2 hrs after the injection. Urination of the animals during death was avoided by ligation of the penis. The organs of interest (liver, kidneys, etc.) and samples of blood and muscles were dissected out and counted in a well-type γ-counter in comparison to a standard. The percentage of the administered dose in the whole organ or tissue was calculated according to the following formula:

$$\% \text{ Dose/organ} = \frac{\text{Counts/min of the sample x Organ weight}}{\text{Counts/min of 1\% Std x Sample Weight}}$$

Blood and skeletal muscles were assumed to be 7% and 43% of the body weight, respectively.

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