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SPECIFIC ASSAY FOR RADIOLABELLED DIGOXIN AND ITS KNOWN APOLAR METABOLITES IN BIOLOGICAL FLUIDS. I.

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SUMMARY

A specific assay method for radiolabelled digoxin and its known apolar metabolites in plasma, urine and saliva was developed. The assay permits the delineation of the pharmacokinetics of digoxin and its metabolites after single-dose administration of the drug to humans. Column chromatographic and solvent extraction procedures were used for the separation of apolar and polar compounds. Thin-layer chromatography was applied for the individual and specific assessment of digoxin and its apolar metabolites. Apolar and polar standards were used for quantitative assessments of all the procedures used. Accuracy and precision of the assay developed were evaluated in plasma, urine and saliva using biological samples spiked with known amounts of standards and by measuring replicates of biological samples obtained from pharmacokinetic studies with digoxin administration to humans.

INTRODUCTION

A large number of methods have been described for the measurement of digoxin in various biological fluids in humans. These methods can be divided in two classes: biochemical and chromatographic assays. Among the biochemical methods there are immunoassays (radio- and enzyme immunoassay [1,2]), procedures based on competitive protein binding [3], inhibition of red cell ^{98}Rb uptake [4] and Na^+,K^+-ATPase [5]. Double isotope dilution derivative assay [6], gas chromatography [7] alone or in combination with mass spectroscopy [8], high-performance liquid chromatography [9], column chromatography and thin-layer chromatography (TLC) combined with extraction procedures [10,11] or mass spectroscopy [12] are the principal chromatographic methods. The biochemical methods are sensitive but not specific per se; the chromatographic methods are claimed to be specific [13]. Unfortunately, the respective sensitivities of the double isotope dilution derivative assay, the high-

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performance liquid chromatographic and gas chromatographic methods are so low as not to permit pharmacokinetic analyses of digoxin after single-dose administration. Column chromatographic and TLC methods could, however, produce both the necessary specifity and sensitivity if a radiolabelled drug is used. The column chromatographic and TLC procedures which have been reported to separate digoxin from its metabolites while possibly being quantitative do not allow assessment of the efficiency of extraction procedures [10, 11].

There is a definite need for pharmacokinetic investigations with digoxin in humans as a function of dose, formulation and route of administration using specific and sensitive methods of analysis. To our knowledge no published studies have been performed to demonstrate the linearity or otherwise of the kinetics of digoxin or of its metabolites [14]. There is conflicting evidence in the literature regarding the extent of metabolism of digoxin. A total of 80—90% of the amounts excreted in the urine was unchanged digoxin after oral administration of the drug in healthy subjects [15—17]. These results contrast with the results of another study, where equal amounts of digoxin and digoxin metabolites were excreted in the urine [18]. An average of 13% (range 1—47%) of the urinary glycosides was dihydrodigoxin assayed by a gas chromatographic method following multiple doses of the drug [19]. However, less than 1% of the urinary excreted total radioactivity represented dihydrodigoxin (assayed following column chromatography) after single-dose administration of radioactively labelled digoxin [15].

Qualitative and quantitative information on the presence of polar, water-soluble metabolites of digoxin is limited. In a recent investigation on the biotransformation of digoxin the polar metabolites were apparently disregarded [15]. In other studies these radioactive metabolites were determined in aqueous residues of urine following chloroform or ethyl acetate extractions in the first 24 h after oral administration of digoxin [20,21]. The proportions ranged between 16 and 20% (chloroform-extracted residue) and 3—30% (ethyl acetate-extracted residue) of the total urinary excretion of radioactivity [20, 21]. Polar, water-soluble metabolites were defined relative to the solvents used for separation in these studies.

Digoxin is a drug with a narrow therapeutic index with overlapping therapeutic and toxic plasma concentrations [22]. Some of its metabolites have been shown to be pharmacologically active in animals [23—25]. The delineation of the kinetics and the extent and type of metabolism of digoxin is crucial for clinical therapy.

We have now developed a specific and sensitive assay to enable measurement of the parent drug and metabolites in plasma, urine and saliva over adequate time periods after single-dose administration of labelled digoxin. The efficiencies of extraction and separation of all the individual steps have been quantified. Labelled apolar and polar standards have been employed to assess the recoveries. Polar metabolites have been separated relative to a known polar standard. The performance, accuracy and precision of the assay have been determined using biological samples spiked with known amounts of labelled standards and also with biological samples obtained from pharmacokinetic studies with administration of digoxin to humans.

EXPERIMENTAL

Material

The following labelled and unlabelled compounds were used and employed as standards: [12α-^{3}H]digoxin (D*), [12α-^{3}H]digoxigenin bisdigitoxoside (DB*), [12α-^{3}H]digoxigenin monodigitoxoside (DM*), digoxin (D), dihydrodigoxin (DH), digoxigenin bisdigitoxoside (DB), digoxigenin (DG), epidoxigenin (EDG), and digitoxin (DT) were donated by Boehringer, Mannheim, G.F.R.; [12α-^{3}H]digoxin-16′-glucuronide (DGL*), [12α-^{3}H]dihydrodigoxin (DH^*_A), [21,22-^{3}H]digitoxin (DT*), digoxin-16′-glucuronide (DGL) were donated by Beiersdorf, Hamburg, G.F.R.; [21,22-^{3}H]dihydrodigoxin (DH^*_B) was provided by Hoffmann-La Roche, Basle, Switzerland. The compounds with the label in the α-position were synthesized according to the method of Von Wartburg et al. [26] and had the following specific activities: D*, 1460 μCi/mg; DB*, 552 μCi/mg; DM*, 457 μCi/mg; DH*, 12772 μCi/mg; DGL*, 435 μCi/mg. DH^*_B was obtained as described elsewhere [27] and had a specific activity of 539 μCi/mg. DT* was synthesized according to the method of Haberland and Maerten [28] and had a specific activity of 539 μCi/mg.

Different TLC systems were set up for the separation of digoxin and its apolar metabolites (TLC systems A—C) and polar metabolites (TLC systems E and F). TLC system D was used for preliminary clean-up of coeluted biological material. The TLC systems were as follows. System A: chloroform developed on Kieselguhr (F_{254}, 5738, 200 μm, Merck, Darmstadt, G.F.R.) pretreated with ethylene glycol (10%, v/v) in acetone in pre-equilibrated chambers. System B: chloroform developed on Kieselguhr (F_{254}, 5738, Merck) pretreated with formamide (8%, v/v) in acetone in pre-equilibrated chambers. System C: dimethyl malonate—propionic acid (3:1) developed on silica gel ($Silicagel_{60}$, F_{254}, 5642, 250 μm, Merck). System D: chloroform—methanol (9:1) developed on silica gel ($Silicagel_{60}$, F_{254}, 5744, 500 μm, Merck). System E: chloroform—methanol (9:1) developed on silica gel ($Silicagel_{60}$, F_{254}, 5715, 250 μm, Merck) pretreated with ethylene glycol (10%, v/v). System F: methanol–water (1:1) developed on silanized silica gel ($Silicagel_{60}$, silanized, F_{254}, 5747, 250 μm, Merck). All the TLC systems except for system C were developed over the whole length of the plates (20 cm); system C was developed over 15 cm only.

The radiochemical purity of D*, DB*, DM*, DH^*_A, DH^*_B, DT* and DGL* was investigated with systems A, B, C, E and F. The results are listed in Table I. For TLC system A separable contaminants with their percentage of retained radioactivity are given in Table II. TLC system A separated DH and DB optimally from D; however, it could not differentiate between DM, DG and EDG. System B assayed D and all of its known apolar metabolites individually, with the exception of the isomers DG and EDG. The separation of DH from D in the latter system was clearly inferior to that of system A. The only advantage of system C was its selective separation of DM from DG and EDG.

DH has been claimed to be a major apolar digoxin metabolite [12,19]. Since optimum selectivity for DH is achieved in TLC system A, this system was employed in the routine analysis of biological samples obtained in the pharmacokinetic studies with D in humans.

The reproducibility of the separation of D and its apolar metabolites DH,

TABLE I

RADIOCHEMICAL PURITY OF LABELLED STANDARDS OF RECOVERED TOTAL RADIOACTIVITY IN TLC SYSTEMS A, B, C, E AND F

Compound	Percentage purity in TLC system				
	A	B	C	E	F
D*	94.8	94.5	97.8		
DH_A^*	100.0				
DH_B^*	97.5	97.2			
DB*	92.5	94.6	93.0		
DM*	97.8	98.4	95.9		
DT*	100.0			100.0	
DGL*				98.5	94.8

TABLE II

CONTAMINANTS OF LABELLED STANDARDS IN PERCENTAGE OF RECOVERED TOTAL RADIOACTIVITY IN TLC SYSTEM A

Compound	Contaminants			
	D*	DH*	DB*	(DM*+DG*+EDG*)
D*	94.8	1.88	2.39	0.92
DH_B^*	2.49	97.5	0	0
DB*	1.44	0.88	92.5	5.20
DM*	0.67	0	1.49	97.8

DB, DG and EDG was studied with system A. An ethanolic solution containing labelled and unlabelled species of the above compounds was spotted on Kieselguhr plates. After development and visualization the respective R_{DH} values for the different compounds relative to DH (R_{DH} = 1.0) were (n = 6): D, 0.85 ± 0.03; DB, 0.58 ± 0.06; DM, DG and EDG, 0.34 ± 0.03.

All organic solvents were of analytical grade.

A commercially available scintillation fluid (Instagel®, Packard-Becker, Groningen, The Netherlands) was used for the counting of radioactivity.

Instruments

The following were used: commercially available columns (Extrelut®, Fertigsäulen, Merck) for column chromatography, various plates for TLC as indicated above, and liquid scintillation spectrometers (Packard Tri-Carb Nos. 3280 and 3255, Packard Instruments, Downers Grove, IL, U.S.A.) for the measurement of radioactivity.

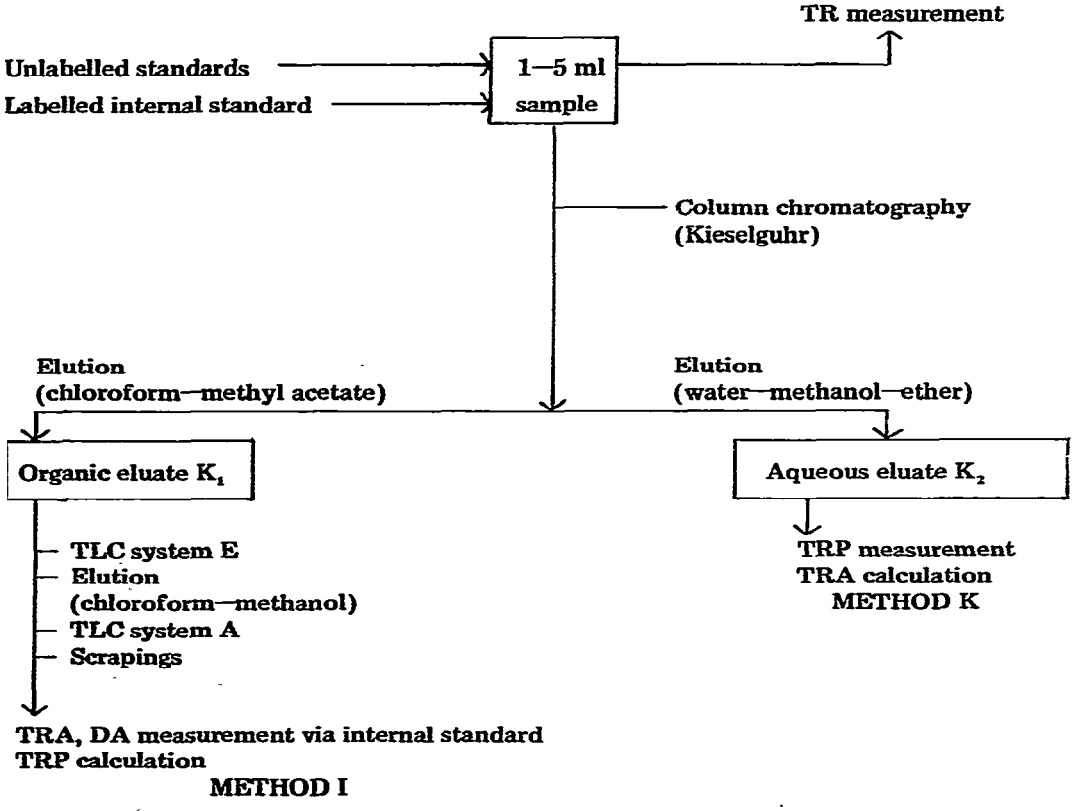
Liquid scintillation counting

Aliquots of biological fluids and their supernatants, eluates, extracts, residues and thin-layer scrapings were transferred into liquid scintillation vials. Eluates and extracts were concentrated to dryness. Then 3.5 ml of water and 10 ml of liquid scintillation fluid were added. After mixing the contents

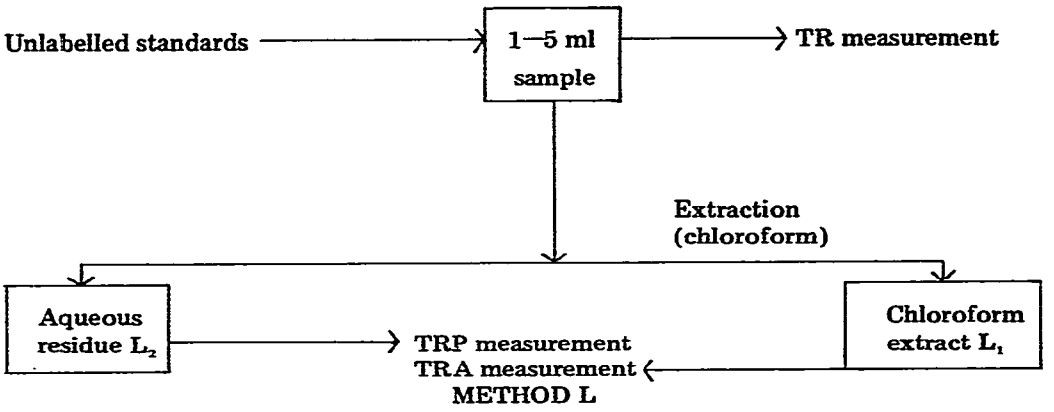
thoroughly, the vials were kept at 37°C for 2 h to enable thorough dissolution with liquid scintillation fluid. Subsequently the vials were kept at 4°C in the dark for several hours prior to counting. The activities measured were corrected for background and quenching using an external standard. Separate quench curves were determined for radioactivity measurements in biological fluids and thin-layer scrapings. Activities of less than twice the background were rejected.

METHODS AND RESULTS

The plus and minus values for mean values in the text refer to the standard deviation (S.D.) of such means; n is the number of experiments performed. The aim of this study was to establish a specific and sensitive assay for (1) labelled D*, (2) its known individual apolar metabolites including DH*, and (3) its (as yet unknown) polar metabolites. The strategy in setting up this assay was to develop three independent methods [methods I, K, and L (Schemes 1 and 2)]: a column chromatographic method (= method K) for the measurement of polar total radioactivity in biological fluids; a solvent extraction method (= method L) for the determination of apolar and polar total radioactivity; a third method (= method I) for the assay of apolar total and individual radioactivity. Method I



Scheme 1. Specific assay for labelled digoxin and metabolites in biological fluids: Flow diagram of methods I and K.



Scheme 2. Specific assay for labelled digoxin and metabolites in biological fluids: Flow diagram of method L.

applied the column chromatographic procedure of method K for separation of apolar and polar total radioactivity followed by specific separation of D, DH, DB and (DM+DG+EDG) by TLC system A. In method I use of DT* as internal standard compensated for losses during the procedure.

Use of unlabelled standards

To ensure constant recoveries, 50 μg unlabelled D, DH, DB, DM, DG, EDG, DGL and DT were routinely added to all the samples prior to processing. Addition of unlabelled standards was also necessary for visualization of the spots on the TLC plates after development. Thus 500 μl of an ethanol—chloroform (1:1) solution containing the standards in a concentration of 100 μg/ml each were placed in glass tubes and subsequently evaporated. Aliquots of the biological samples to be processed were then added.

Measurement of total radioactivity in plasma, urine and saliva

Duplicates of 100—500 μl of plasma, 100 μl of urine and 100—300 μl of pre-centrifuged saliva were assayed and the means used for calculation of the respective concentrations. Saliva samples were centrifuged at 300 *g* for 10 min prior to assay. Radioactivity was subsequently measured in the clear supernatant devoid of mucus. In preliminary experiments fresh saliva was spiked with known amounts of D* and after centrifugation the percentage recovery of the apolar standard, D*, in the supernatant, $r^{s'}_{D*}$, was determined: $r^{s'}_{D*} = 86.6 \pm 3.8\%$ ($n = 4$). Total radioactivity in saliva, TR^s, was then calculated from total radioactivity in saliva supernatant, $TR^{s'}$, on the assumption that the recovery of total radioactivity in saliva supernatant, $r^{s'}_{TR}$, was similar to that of D*, i.e. $r^{s'}_{TR} \approx r^{s'}_{D*}$.

Method I

In this procedure DT* was used as an internal standard, to measure the respective apolar total and individual radioactivities in biological fluids, TRA^{bf} and DA^{*bf} [$= D^{*bf} + DH^{*bf} + (DM^*+DG^*+EDG^*)^{bf}$]. Polar total radioactiv-

ity in biological fluids, TRP^{bf}, was a calculated value with method I and was obtained from the difference between the experimentally measured total radioactivity in a sample, TR^{bf}, and its total apolar radioactivity, TRA^{bf}.

DT* dissolved in the corresponding biological fluid was added as internal standard to aliquots of 1 or 5 ml of plasma or 1 ml of urine or saliva. DT* radioactivity approximated the inherent total radioactivity of the sample. At the same time an aliquot of DT* as internal standard was transferred to a liquid scintillation vial for measurement of its radioactivity. The biological samples were then diluted with water to total volumes of 20 ml and placed on the Kieselguhr columns according to method K as described below. Eluate 1 containing the apolar D*, DH^*_B, DB*, DM* and DT* was evaporated under vacuum at 40°C and redissolved in 0.5 ml of chloroform—ethanol (1:1). Aliquots of 200—500 µl of this fraction were added in bands of 6 cm on preparative silica gel plates and developed in TLC system D. This was effected by means of pipettes ("Konstriktionspipetten", Becton-Dickinson, Rutherford, NJ, U.S.A.), which had specially prepared polyethylene tubings set over their tips. This first TLC step proved to be necessary for the definitive elimination of coeluted biological material which hindered an effective TLC separation of D and its apolar metabolites (DH, DB, DM, DG, EDG). The silica gel plates of system D were developed as described above.

For reversible visualization of the TLC area containing the glycosides the plates were sprayed with a 1% ethanolic solution of iodine for 30 sec. In preliminary experiments it was shown that the 1% ethanolic solution of iodine did not induce degradation of the standards with loss of the label. Two clearly separated areas were apparent on the plates: one containing the glycosides, the other eluted biological contaminants. The latter part of the plate was cut out and the remaining part was then subjected to an elution procedure for 24 h. Slices (10 cm) of filter paper were used: one end was attached to the upper part of the plate by clamps, the other end reached into a sink containing a solution of chloroform—methanol (1:1). In preliminary experiments the efficiency of this elution procedure was checked at different periods of time. Elution was considered to be complete when spraying with the 1% ethanolic solution of iodine did not reveal any visible spots on the plates. This was accomplished in less than 24 h. The eluates thus obtained were then evaporated under vacuum at 40°C and redissolved in 0.5 ml of chloroform—ethanol (1:1). Aliquots (20—500 µl) of this solution were added to Kieselguhr plates by means of Lambda pipettes (Clay Adams Co., New York, NY, U.S.A.) or added as bands by means of pipettes equipped with specially prepared polyethylene tubings. The plates of system A were then developed as described above. After development the plate areas adjacent to the solvent front were first scraped off. These areas contained the internal standard DT*. When aliquots of 500 µl had been added as bands, rectangular sections (2.5 × 10 cm) were scraped off for radioactivity determination; when aliquots of 20—50 µl had been added as spots, squares of 2.5 × 2.5 cm were scraped off for radioactivity determination. The parts of the plates containing the unlabelled and labelled D, DH, DB, (DM+DG+EDG) and DGL were then heated at 110°C for 5 min and the spots were visualized by spraying the plates with 1% ethanolic iodine solution. Removal of the internal standard DT* from the plates prior to the visualization

procedure proved to be necessary. Preliminary experiments had shown that the DT* employed was thermolabile (contrary to all the other labelled standards used). In other experiments it was demonstrated that the scraped areas contained reproducibly all the DT* present on the plates. In further experiments the relative sizes of the spots of the labelled and unlabelled species of the standard compounds D, DH, DB, (DM+DG+EDG) and DGL were compared. Systematic measurements of the radioactivity from 1-mm scrapings showed that the areas which contained the labelled species exceeded clearly those that contained the visualized fraction of the unlabelled species. The visualized spots and bands were expanded, respectively, to 2 × 2 cm squares and 10 × 2 cm rectangular sections. Such scrapings yielded radioactivity peaks for the individual standard compounds similar to those obtained from the scrapings of 1-mm bands. In the routine procedure the visualized spots and bands were expanded as described and marked accordingly. Prior to scraping the plates were heated again for 2 min at 110°C to eliminate remaining traces of iodine, since iodine was shown to be a potent quencher of radioactivity. The scrapings (2-mm bands) were then individually transferred to liquid scintillation vials and their radioactivity counted. The radioactive distribution pattern was plotted by a computer plotter programmed to give the total radioactivity scraped from the plates, the number of radioactivity peaks, their R_F values and the percentage of radioactivity in each peak.

The accuracy and precision of method I were tested by spiking plasma, urine and saliva with a solution containing known relative radioactivities of D* (59.5%), DH^*_B (6.22%), DB* (7.61%), DM* (6.14%) and DLG* (20.51%). Similar percentage radioactivities of the individual compounds were expected to occur in biological samples after administration of digoxin to humans [20,21]. The internal standard, DT*, had a radioactivity equal to the total radioactivity of the labelled digoxin derivatives. Aliquots of fresh plasma (1 and 5 ml), urine (1 ml) and saliva (1 and 5 ml) were spiked, subsequently assayed by method I as described above and the recoveries for the individual apolar radioactivity, DA^{*bf}_I, total apolar radioactivity, TRA^{bf}_I, and total polar radioactivity, TRP^{bf}_I, determined (see Appendix). Percentage recoveries (accuracies) and deviations (precisions) for DA^{*bf}_I, TRA^{bf}_I and TRP^{bf}_I as obtained by method I in plasma, urine and saliva are listed in Table III.

The percentage recoveries of the experimentally measured DA^{*bf}_I and TRA^{bf}_I, $r^{bf}_{I,DA*}$ and $r^{bf}_{I,TRA}$, were, except for $r^{bf}_{I,DH*}$, in the range 91—113% in all the biological fluids tested (Table III). $r^{P}_{I,DH*}$ and $r^{u}_{I,DH*}$ deviated more and were 55% and 119%, respectively. The percentage recoveries of the calculated TRP^{bf}_I, $r^{bf}_{I,TRP}$, differed more from 100% from those of the experimentally measured TRA^{bf}_I, $r^{bf}_{I,TRA}$ (Table III). It was concluded that method I was most adequate for measuring DA^{*bf}_I and TRA^{bf}_I reliably with reservations regarding DH*. The results obtained for TRP^{bf}_I suggested that these calculated values were less reliable and more biased.

Methods K and L

Methods K and L were set up for a direct measurement of polar total radioactivity in biological fluids, TRP^{bf}. Apolar total radioactivity in biological fluids, TRA^{bf}, was a measured value with method L and a calculated value with

method K. TRA_K^{bf} was obtained from the difference between the experimentally measured total radioactivity in a sample, TR^{bf}, and its polar total radioactivity, TRP_K^{bf}, with method K.

The values for TRP^{bf} and consequently for TRA^{bf} (so obtained) respectively by methods K and L were not expected to be identical, since the separation procedure of the two methods differs.

Individual apolar radioactivity, DA^{*bf}, could only be assessed by methods K and L if the relative radioactivities for D*, DH*, DB* and (DM+DG+EDG)* simultaneously obtained by method I were used.

Method K

Columns (8 × 2.5 cm) were packed with Kieselguhr of 0.5 mm particle diameter (Extrelut®, Fertigsäulen, Merck). Aliquots of 1 ml of urine and saliva and 1—5 ml of plasma were diluted with water to total volumes of 20 ml and then immediately applied on the columns. The first elution was carried out with 40 ml of chloroform—methyl acetate (1:4) (flow-rate, 0.4 ml/min); 25 ml of eluate 1 containing the apolar digoxin and metabolites were obtained and processed as described above (method I). The second elution obtained on passage of 40 ml of water—methanol—diethyl ether (1:1:1) yielded eluate 2 containing the polar metabolites (flow-rate, 0.2 ml/min). Eluate 2 was then concentrated to dryness by evaporation at 40°C under vacuum, redissolved in 3 ml of methanol and transferred to a liquid scintillation vial. The radioactivity was measured after concentrating it to dryness under a stream of air.

In preliminary experiments the separation and elution efficiency of method K was tested with the apolar and polar standards D* and DGL*, respectively. Aliquots of 1, 2, 3 and 5 ml of plasma and 1 ml of urine were spiked with known amounts of D* and DGL* in separate experiments, and the recoveries determined (see Appendix). The percentage recovery of D* in eluate 2 for 1, 2, 3 and 5 ml of plasma was 2.2 ± 0.40% ($n = 6$) and was volume-independent. The percentage recovery of DGL* for 1, 2, 3 and 5 ml plasma was 83.9%, 81.0%, 69.3% and 56.8%, respectively, and was volume-dependent (two-tailed paired t-test: t (0.05) = 2.02, $t_{cal} = 13.64$). The percentage recovery of D* and DGL* in eluate 2 of urine, was 2.3% and 89.5%, respectively. These results indicate that separation of apolar and polar total radioactivity in eluate 2 was not complete and that the measured activity represented total radioactivity, $TR^{K_2,bf}$.

The accuracy and precision of method K were tested by spiking aliquots of plasma, urine and saliva with a solution containing known amounts and radioactivities of D*, DH_B^*, DB*, DM* and DGL* as described above (see method I). Samples of fresh plasma (1 and 5 ml), urine (1 ml) and saliva (1 and 5 ml) were spiked with these standards, carried through method K as described above, and the recoveries of DA^{*bf}, TRA^{bf} and TRP^{bf} were then determined. DA^{*bf} was assessed by combining data obtained by methods I and K (see Appendix). The percentage recoveries (accuracies) and deviations (precisions) for TRP_K^{bf}, TRA_K^{bf} and $DA^{*bf}_{I/K}$ as obtained by method K in plasma, urine and saliva are listed in Table IV.

The percentage recoveries of the experimentally measured TRP_K^{bf}, $r_{K,TRP}^{bf}$, ranged between 84 and 116% in the three biological fluids studied (Table IV).

TABLE III

DETERMINATION OF PERCENTAGE RECOVERIES OF APOLAR TOTAL AND INDIVIDUAL RADIOACTIVITY AND OF POLAR TOTAL RADIOACTIVITY IN BIOLOGICAL SAMPLES BY METHOD I

Plasma, urine and saliva samples were spiked with known amounts and relative radioactivities of D*, DH^*_B, DB*, DM* and DGL*.

Biological fluid	Volume (ml)	$r^{bf}_{I,TRA}$		$r^{bf}_{I,D*}$		$r^{bf}_{I,DH*}$		$r^{bf}_{I,DB*}$		$r^{bf}_{I,DM*}$		$r^{bf}_{I,TRA}$		n
		Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	
Plasma	1/5	95.4	1.61	99.5	2.08	54.9	1.45	93.5	1.50	99.2	1.29	118.5	6.68	4
Urine	1	112.1	3.91	113.0	3.36	118.7	6.01	103.1	3.98	106.9	7.96	53.3	15.1	4
Saliva	1/5	97.7	1.70	98.2	1.36	103.2	6.34	91.4	2.70	94.9	2.68	109.1	6.58	4

TABLE IV

DETERMINATION OF PERCENTAGE RECOVERIES OF APOLAR AND POLAR TOTAL RADIOACTIVITY IN BIOLOGICAL SAMPLES BY METHOD K AND OF APOLAR INDIVIDUAL RADIOACTIVITY BY METHODS I AND K COMBINED

Plasma, urine and saliva samples were spiked with known amounts and relative radioactivities of D*, DH^*_B, DB*, DM* and DGL*.

Biological fluid	Volume (ml)	$r^{bf}_{K,TRA}$		$r^{bf}_{I/K,D*}$		$r^{bf}_{I/K,DH*}$		$r^{bf}_{I/K,DB*}$		$r^{bf}_{I/K,DM*}$		$r^{bf}_{K,TRP}$		n
		Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	
Plasma	1	96.0	1.06	100.5	1.07	53.8	0.29	92.1	1.03	100.0	1.81	115.6	4.12	2
	5	100.1	0.85	104.3	0.23	59.3	1.00	100.4	2.10	104.3	2.49	99.6	3.31	2
Urine	1	102.5	0.54	103.4	0.88	108.6	2.19	94.3	0.89	97.7	7.58	90.3	2.09	4
Saliva	1	104.2	0.87	104.7	0.87	110.0	5.86	97.4	1.98	101.2	2.99	83.9	3.38	4

The percentage recoveries of the calculated TRA_K^{bf} and $DA^{*bf}_{I/K}$, $r^{bf}_{K,TRA}$ and $r^{bf}_{I/K,DA^*}$ respectively, were even closer to 100% and were (except for $r^{bf}_{I/K,DH^*}$) in the range 92—105% (Table IV). The precision of method K for TRP^{bf} and TRA^{bf} appeared to be superior to that of method I. It was concluded that method K was suitable for assessment of TRP^{bf} and TRA^{bf}.

Method L

A volume of 1 ml of plasma, urine or saliva was diluted with water to a total volume of 5 ml and then extracted three times with chloroform. Plasma was extracted with five times the volume of chloroform and urine and saliva with three times the volume of chloroform. These volume ratios were adequate for the clear separation of the organic and aqueous phases with minimal emulsion. The chloroform extracts were then combined and 1- or 2-ml aliquots were transferred to liquid scintillation vials. The radioactivity was determined after concentration under a stream of air to dryness. Aliquots of 1—3 ml of aqueous residues were transferred to liquid scintillation vials and the radioactivity determined after the remaining chloroform had been eliminated.

In preliminary experiments the extraction efficiency of method L was tested with the apolar and polar standards D* and DGL*, respectively. Aliquots of 1 ml of plasma or urine were spiked with known amounts of D* and DGL* in separate experiments and the respective recoveries determined (see Appendix).

The percentage recoveries were: in plasma for D* (n = 2), in eluate 1 = 98.9, 97.1%, in eluate 2 = 1.1, 2.9%; and for DGL* (n = 2) in eluate 1 = 2.5, 3.3% and eluate 2 = 97.5, 97.1%. The corresponding percentage recovery values for D* in urine were (n = 2), in eluate 1 = 98.9, 98.8%, in eluate 2 = 1.1, 1.2%; and for DGL* (n = 2), in eluate 1 = 2.8, 2.5%, and in eluate 2 = 97.2, 97.5%.

The extraction efficiency of D* and DGL* in saliva with method L was not determined and was assumed to be identical to that in urine.

The accuracy and precision of method L were tested by spiking plasma, urine and saliva with a solution containing known amounts and relative radioactivities of D*, DH^*_B, DB*, DM* and DGL*, as described above (see method I). Samples of fresh plasma (1 and 5 ml), urine (1 ml) and saliva (1 and 5 ml) were spiked with those standards, carried through the procedure of method L as described above, and the recoveries of DA^{*bf}, TRA^{bf} and TRP^{bf} were then determined. The extraction efficiency obtained for D* and DGL* in preliminary experiments showed that the measured radioactivities in both the aqueous residue, L_2, and the organic extract, L_1, of a biological sample represented total radioactivity, $TR^{L_2,bf}$ and $TR^{L_1,bf}$, respectively. DA^{*bf} was assessed by combining data obtained by methods I and L (see Appendix).

Percentage recoveries (accuracies) and deviations (precisions) for TRP^{bf}_L and TRA^{bf}_L in the biological samples as obtained by method L in plasma, urine and saliva are listed in Table V. Percentage recoveries for $DA^{*bf}_{I/L}$ are also included.

The respective percentage (accuracies) of the experimentally measured TRP^{bf} and TRA^{bf}, $r^{bf}_{L,TRP}$ and $r^{bf}_{L,TRA}$, were close to 100% and ranged between 99 and 105% in all three biological fluids studied (Table V). The percentage recoveries of DA* (except for DH*) ranged between 92 and 101%. The precision of TRA^{bf} assessments by method L appeared to be similar to that by

TABLE V

DETERMINATION OF PERCENTAGE RECOVERIES OF APOLAR AND POLAR TOTAL RADIOACTIVITY IN BIOLOGICAL SAMPLES BY METHOD L AND OF APOLAR INDIVIDUAL RADIOACTIVITY BY METHODS I AND L COMBINED

Plasma, urine and saliva samples were spiked with known amounts and relative radioactivities of D*, DH^*_B, DB*, DM* and DGL*.

Biological fluid	Volume (ml)	$r^{bf}_{L,TRA}$		$r^{bf}_{I/L,D*}$		$r^{bf}_{I/L,DH*}$		$r^{bf}_{I/L,DB*}$		$r^{bf}_{I/L,DM*}$		$r^{bf}_{L,TRP}$		n
		Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	Mean (%)	S.D.	
Plasma	1	99.5	2.06	98.6	0.48	54.4	1.75	100.2	2.56	100.4	0.70	101.9	7.89	2
Urine	1	100.2	3.07	101.1	0.64	106.1	2.03	92.2	1.23	95.5	4.10	99.2	1.29	2
Saliva	1	98.8	1.30	99.2	0.65	104.3	5.11	92.5	1.06	95.9	2.49	104.8	5.03	2

TABLE VI

PRECISION* OF METHODS I, K AND L ASSESSED FROM REPLICATE MEASUREMENTS OF APOLAR AND POLAR TOTAL RADIOACTIVITY IN BIOLOGICAL SAMPLES OBTAINED FROM A PHARMACOKINETIC STUDY WITH DIGOXIN ADMINISTRATION TO HUMANS

Median, (range), and number of samples with replicate measurements (n) are given.

Biological fluid	TR^{bf}	TRA^{bf}			TRP^{bf}			D^{*bf}	DH^{*bf}	DB^{*bf}	DM^{*bf}**
		I	K	L	I	K	L	I***			
Plasma	1.4 (0.3—12) n=17	2.7 (1.1—16)	4.5 (0.1—52) n=17	17 (0.5—15)	140 (0.1—140)	79 (4.2—140) n=17	13 (0.5—65)	0.5 (0.1—5.4)	15 (1.1—71) n=16	5.2 (0.1—67)	20 (0.1—110)
Urine	2.4 (0.4—10) n=13	2.4 (0.1—6.8) n=13	1.9 (0.1—3.5) n=7	0.6 (0.3—1.1) n=7	66 (0.1—140) n=13	21 (0.1—140) n=7	5.4 (1.7—67) n=7	0.1 (0.1—0.7)	10 (1.3—47) n=13	7.1 (0.1—20)	13 (0.1—50)

*Precision is defined as percentage standard deviation about the mean of replicate measurements.

**$DM^{*bf} = (DM+DG+EDG)^{*bf}$.

***Estimated from replicate measurements of $DA^{*I,bf}/TRA^{I,bf}$.

methods I and K. The precision of TRP^{bf} estimates by method L was closer to that by method K (Table V). It was concluded that method L was adequate for assessments of TRP^{bf} and TRA^{bf}.

Stability and radiolysis

Four plasma and urine aliquots (20 ml) were spiked with known amounts of D*. The D* used had the known percentage impurities [DH* (1.9%), DB* (2.4%), (DM+DG+EDG)* (0.9%)] previously determined with TLC system A. The spiked plasma and urine aliquots were deep-frozen and kept at −20°C for 1—10 months. The aliquots underwent different treatments. The first pair of plasma and urine aliquots was thawed and subsequently refrozen four times at 1, 4, 7 and 10 months after spiking. The second pair of aliquots was thawed and refrozen three times at 1, 4 and 7 months after spiking. The third pair of aliquots was thawed and refrozen twice at 1 and 4 months after spiking, and the fourth pair was thawed after it had been kept frozen for 1 month. After thawing all the aliquots were kept at room temperature for 8 h and samples were then taken. They were processed according to method K.

The percentages of recovered total radioactivity, TR^{bf}, which really represented total apolar radioactivity, TRA^{bf}, were time-independent and constant and were on the average for plasma and urine 97.9 ± 5.1% (n = 10) and 96.2 ± 4.9% (n = 11), respectively. These values agreed with the respective 97.8% and 97.7% which optimally could be expected on the basis of the data of preliminary experiments in which plasma and urine aliquots had been spiked with D* and its recovery had been determined in eluate K_2. Of the recovered TRA^{bf} constantly 95.3 ± 0.46% (n = 10) was D*, 1.4 ± 0.36% (n = 10) was DH*, 2.4 ± 0.15% (n = 10) was DB* and 0.96 ± 0.14% (n = 10) was (DM+DG +EDG)* in plasma. In urine the corresponding figures were: 94.5 ± 0.76% (n = 11) was D*, 2.7 ± 0.20% was DH*, 2.4 ± 0.09% (n = 11) was DB* and 0.82 ± 0.09% was (DM+DG+EDG)*. It was concluded that the tested compounds were stable for at least ten months at −20°C including repeated thawing and exposure to ambient temperature.

Identical studies were performed with DGL* in plasma and urine to investigate a possible deconjugation of this compound. The DGL* used had a known percentage impurity (D* 1.5%, 5.2%) previously determined with TLC systems E and F. TR^{bf} was determined in eluates K_1 and K_2 according to method K (see Scheme 1) except that eluate K_1 was obtained by using 40 ml of methyl acetate instead of chloroform—methyl acetate (1:4). The percentages of recovered TR^{bf} in the experiments with plasma and urine were on the average 102.2 ± 4.5% (n = 10) and 101.7 ± 2.8 (n = 10) respectively. 2.2 ± 0.38% (n = 10) and 2.5 ± 0.79% (n = 10) respectively of TR^{bf} were recovered constantly and time independently in the apolar eluate K_1 with plasma and urine. It was concluded that hydrolysis of DGL* did not occur for at least ten months at −20°C including repeated thawing and exposure to ambient temperature.

Precision of methods I, K and L assessed from replicate measurements of unknown radioactivity in biological samples obtained from pharmacokinetic studies

Replicate determinations of TRA^{bf} and TRP^{bf} in plasma and urine were performed by methods I, K and L. The samples were obtained from two healthy male subjects to which [^{3}H]digoxin had been administered intravenously (0.6 mg to H.R., 1.2 mg to R.F.). The samples chosen covered the whole concentration range of radioactivity found after intravenous dosing of the labelled drug. Unknown DA^{*bf}, TRA^{bf} and TRP^{bf} in biological samples were determined by method I by measuring TR^{bf} and $DA^{*I,bf}$ ($TRA^{I,bf}$). These values were corrected by the individual recoveries of DA^{*bf}, TRA^{bf} and TRP^{bf} previously determined with spiked plasma, urine and saliva samples (see Appendix). Unknown TRP^{bf} and TRA^{bf} in biological samples were determined by method K by assaying TR^{bf} and $TR^{K_2,bf}$ (see Appendix). These values were corrected by the individual recoveries of TRA^{bf} and TRP^{bf} previously determined with spiked plasma, urine and saliva samples. Unknown TRP^{bf} and TRA^{bf} in biological samples were determined by method L by measuring $TR^{L_2,bf}$ and $TR^{L_1,bf}$ and considering the previously found individual recoveries of DA^{*bf}, TRA^{bf} and TRP^{bf} with spiked plasma, urine and saliva samples (see Appendix). Replicate determinations were also made of $DA^{*I,bf}/TRA^{I,bf}$ by method I and of TR^{bf} by direct measurement.

Precision was defined as percentage standard deviation about the means of replicate measurements. Medians and ranges of precision for TRA^{bf} and TRP^{bf} obtained by the different methods and for TR^{bf} obtained by direct assay are listed in Table VI. The estimated precisions of TR^{bf} and TRA^{bf} determined directly and by methods I, K and L were similarly high in both biological fluids studied (Table VI). The precisions of TRP^{bf} were high for method L only and were significantly lower for methods I and K in plasma and urine. The precisions of $DA^{*I,bf}$ decreased in the order $D^* > DB^* > DH_B^* > DM^*$, and the individual values for the compounds susggested that at least D^{*bf} and DB^{*bf} measurements were sufficiently precise (Table VI). It was concluded that method L displayed the highest overall precision and was the only method in which reproducible estimates of TRP^{bf} in plasma and urine were obtained. Methods I, K and L were considered to be equally precise regarding TRA^{bf} measurements. The comparatively lowest precision of TRP^{bf} obtained by method I was considered to result from the fact that after intravenous administration the percentage of total polar radioactivity was small [$10^2 \times (TRP^{bf}/TR^{bf}) < 5\%$] in both plasma and urine. The value of TRP_I^{bf} was calculated and obtained from the difference of two values of almost equal size ($TR^{bf} \approx TRA^{bf}$). Estimates of TRP^{bf} by method L have a higher precision than those by method K. This could be due to the fact that with method L both TRA^{bf} and TRP^{bf} were measured (and normalized) values, whereas with method K only TRP^{bf} was a measured value, but TRA^{bf} a calculated value. Alternatively, the column chromatographic procedure of method K (and method I) could have produced more variable estimates of TRP than the chloroform extraction procedure of method L.

Correlations of apolar and polar total radioactivity determinations by methods I, K and L in plasma and urine

It appeared to be useful to study the nature of existing relationships between methods I, K and L regarding their respective assessments of TRA^{bf} and TRP^{bf}. Knowledge of the nature of such interassay relationships could give valuable information on the individual characteristics and possible biases of the methods used. Correlations between the different TRA^{bf} values and between the different TRP^{bf} values determined by methods I, K and L in plasma and urine were attempted. The plasma and urine samples for this investigation had been obtained from volunteer R.F. who had received four dosages of [^{3}H]-digoxin on separate occasions: 1.2 mg and 0.6 mg intravenously as well as orally. The correlations were performed with the data sets of each study: separately for TRA^{bf} and TRP^{bf} and separately for these values in plasma and urine. No attempt was made to correlate TRP^{bf} data obtained by the three methods in the intravenous studies. The precision of TRP^{bf} measurements by methods I and K (Table VI) was considered to be too low.

Linear regressions of the type $y = mx + c$ were attempted, where y and x correspond to the TRA^{bf} (or TRP^{bf}) data sets obtained by two different methods and where m and c represent slope and intercept, respectively. A statistical program [29] was used which yielded correlation coefficients, slopes and intercepts. Both variables were assumed to be normally distributed. The means of the variances for TRA^{bf} and TRP^{bf} measurements by methods I, K and L, obtained previously from replicate determination of samples by these methods, were used as variability estimates. Coefficients of correlations were also calculated according to non-parametric statistical procedures [30].

Highly significant linear correlations existed between the TRA^{bf} values (y,x) and between the $TRP^{bf}(y,x)$ values assayed by methods I, K and L in plasma and urine at both dosage levels

$$y_K^{bf} = mx_I^{bf} + c \quad (1)$$

$$y_L^{bf} = mx_I^{bf} + c \quad (2)$$

$$y_L^{bf} = mx_K^{bf} + c \quad (3)$$

The parameters of eqns. 1—3 are given for TRA^{bf} and TRP^{bf}, respectively, in Tables VII and VIII. For TRA^{bf} the slopes of all regressions were close to 1.0 and the intercepts negligible [not withstanding the fact that in some cases slopes and intercepts were statistically significantly different from 1.0 and 0, respectively (Table VII)]. Methods L and K gave practically identical values and small interassay variability in plasma and urine. TRA^{bf} estimates by these methods tended to be higher than those by method I. Regressions between TRP^{bf} values by method L or K and by method I yielded slopes that were clearly smaller than 1.0 and intercepts that were close to zero (Table VIII). TRP^{bf} estimates by method I were thus larger than those by methods K or L. TRP^{bf} values by methods L and K were equivalent in urine, whereas in plasma method K tended to give smaller values than method L.

It was concluded that method I estimates of TRP^{bf} and TRA^{bf} differed from those by methods K and L. This discrepancy was pronounced for the TRP^{bf}

TABLE VII

LINEAR REGRESSIONS OF APOLAR TOTAL RADIOACTIVITY DETERMINATIONS IN PLASMA AND URINE BY METHODS I, K AND L ($TRA^{bf}_{I,K,L}$)

Dose (mg)	Mode of administration	Fluid	n*	Range (dpm/ml)	Methods	m**	S.D.	c***	± S.D.	r§
1.2	Intravenous	Plasma	32	365,120—12,146	K / I	0.997*	0.0059	−23.0	238.7	1.000
					L / I	0.997*	0.0066	130	263.7	0.999
					L / K	0.980*	0.0022	159	88.5	1.000
		Urine	13	127,299—0	K / I	1.058*	0.0091	−2005	2408	1.000
					L / I	1.059*	0.0081	−3432	2154	1.000
					L / K	1.000	0.0023	−1391	655.0	1.000
0.6	Intravenous	Plasma	32	478,530—22,892	K / I	1.076*	0.0110	−377	308.4	0.999
					L / I	1.063*	0.0101	354	284.8	0.999
					L / K	0.987*	0.0034	33.6	102.1	1.000
		Urine	13	116,470—0	K / I	1.027	0.0229	609.5	5010	0.997
					L / I	1.021	0.0231	739.2	5050	0.997
					L / K	0.992*	0.0019	453.7	421.9	1.000
1.2	Oral	Plasma	27	544,744—22,930	K / I	1.235*	0.0163	−366*	78.3	0.998
					L / I	1.201*	0.0165	−342*	79.3	0.990
					L / K	0.971*	0.0087	17.5	49.8	0.990
		Urine	13	16,110—0	K / I	1.127*	0.0268	−5853	5450	0.997
					L / I	1.133*	0.0254	−5985	5177	0.997
					L / K	1.004	0.0162	—332.9	1157	1.000
0.6	Oral	Plasma	27	310,367—20,802	K / I	1.002	0.0107	20.6	50.7	0.999
					L / I	0.969*	0.0091	34.6	43.1	0.999
					L / K	0.967*	0.0043	52.2*	20.4	1.000
		Urine	13	12,510—75	K / I	1.056*	0.0220	−1463	3155	0.998
					L / I	1.059*	0.0219	−1797	3142	0.998
					L / K	1.001	0.0039	−150.8	583.1	1.000

*Number of pairs.
**Slope (*significantly different from 1.0, $p < 0.05$).
***Intercept (*significantly different from zero, $p < 0.05$).
§ Correlation coefficient.

TABLE VIII

LINEAR REGRESSIONS OF POLAR TOTAL RADIOACTIVITY DETERMINATIONS IN PLASMA AND URINE BY METHODS I, K AND L ($TRP^{bf}_{I,K,L}$)

Dose (mg)	Mode of administration	Fluid	n*	Range (dpm/ml)	Methods	m**	±	S.D.	c***	±	S.D.	r§
1.2	Oral	Plasma	26	2153—0	K / I	0.361*		0.050	184		77.6	0.811
					L / I	0.607*		0.080	88.7		116	0.826
					L / K	1.282*		0.044	−11.6		32.2	0.986
		Urine	13	114,410—0	K / I	0.519*		0.064	2602		3144	0.921
			13		L / I	0.528*		0.063	1918		3073	0.927
			13		L / K	0.980*		0.041	89.1		1146	0.990
0.6	Oral	Plasma	27	2273—0	K / I	0.759*		0.047	53.3		30.3	0.954
					L / I	1.075		0.083	99.0		51.6	0.932
					L / K	1.331		0.033	49.6*		17.2	0.993
		Urine	13	39,864—0	K / I	0.756		0.146	−1243		2864	0.817
			13		L / I	0.832		0.171	−2441		3271	0.814
			13		L / K	1.007		0.038	−94.0		531	0.992

*Number of pairs.

**Slope (*significantly different from 1.0, $p < 0.05$).

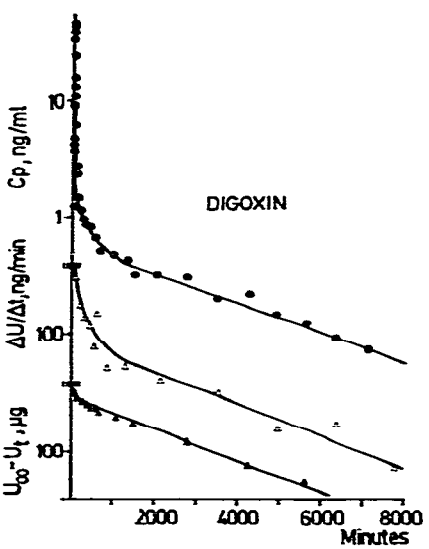
***Intercept (*significantly different from zero, $p < 0.05$).

§ Correlation coefficient.

values and was probably a consequence of the fact that with method I TRP^{bf} was a calculated value, obtained from the difference of two similarly large values, TR^{bf} and TRA^{bf}. The smaller discrepancies between the experimentally measured TRP^{bf} values by methods K and L were most likely due to the use of different separation procedures. It was concluded that method I estimates of TRP^{bf} (and TRA^{bf}) were more biased than those by methods K and L. Method L which, unlike the other two methods, determines both TRA^{bf} and TRP^{bf} experimentally, was regarded as the most adequate assay. In conclusion, method L in combination with method I appears to be the best procedure for the determination of digoxin and its apolar and polar metabolites in biological samples obtained from humans after administration of radiolabelled digoxin.

DISCUSSION

The assay methods presented allow a complete pharmacokinetic analysis of digoxin and its metabolites in biological fluids in humans after single-dose administration of the drug (Figs. 1—6). They provide the necessary specificity and, if radiolabelled drug is used, sensitivity. Parent drug and apolar individual



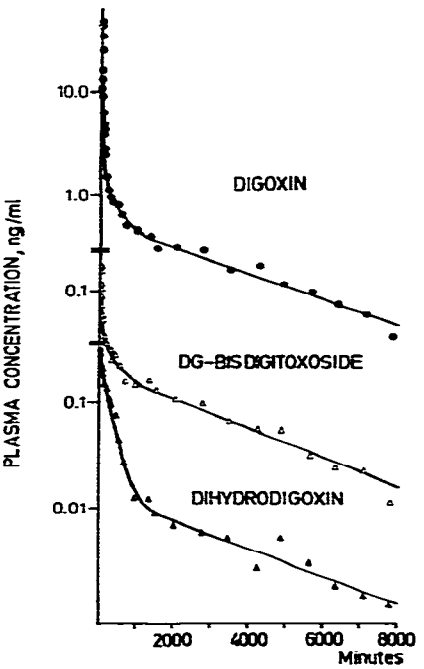


Fig. 1. Typical semilogarithmic plots of plasma concentrations of digoxin, C_p (●), rates of urinary excretion of digoxin, $\Delta U/\Delta t$ (△), and amounts of digoxin yet to be excreted, ($U_\infty - U_t$) (▲), against time for intravenous administration (0.6 mg of [^{3}H]digoxin to H.R.). Data were obtained by method L in combination with method I.

Fig. 2. Typical semilogarithmic plots of plasma concentrations of digoxin (●), digoxigenin bisdigitoxoside (△) and dihydrodigoxin (▲) against time for intravenous administration (0.6 mg of [^{3}H]digoxin to H.R.). Data were determined by method L in combination with method I.

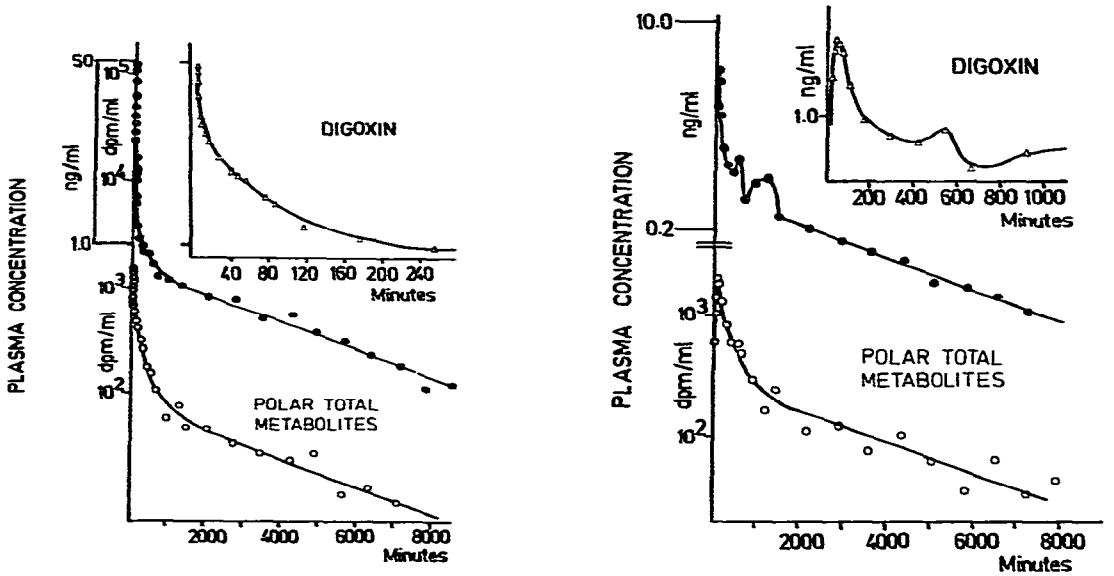


Fig. 3. Typical semilogarithmic plots of plasma concentrations of digoxin (●) and polar total metabolites (○) against time for intravenous administration (0.6 mg of [^{3}H]digoxin to H.R.) (△). Inset: plasma levels of digoxin on an expanded time scale. Data were obtained by method L in combination with method I.

Fig. 4. Typical semilogarithmic plots of plasma concentrations of digoxin (●) and polar total metabolites (○) against time for oral administration (0.6 mg of [^{3}H]digoxin to R.F.) (△). Inset: plasma levels of digoxin on an expanded time scale. Data were determined by method L in combination with method I.

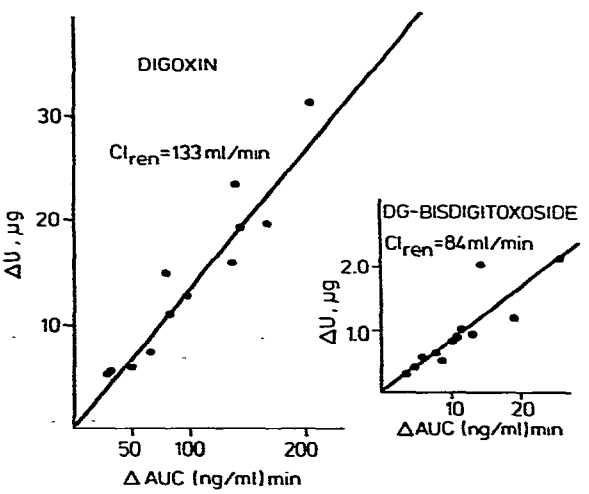


Fig. 5. Typical linear renal clearance plots for digoxin and digoxigenin bisdigitoxoside (inset) after oral administration of 0.6 mg of [^{3}H]digoxin to R.F. The values of the renal clearance, Cl$_{ren}$, were obtained from the slopes of these plots. Data were assayed by method K in combination with method I.

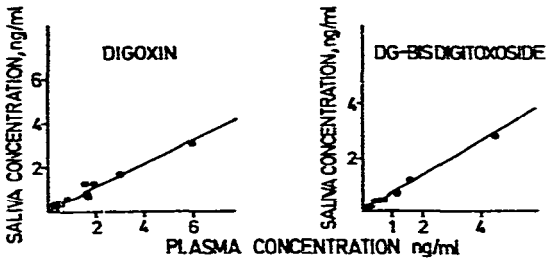


Fig. 6. Typical linear plots of saliva concentrations against plasma concentrations for digoxin and digoxigenin bisdigitoxoside after intravenous administration of 1.2 mg of [^{3}H]digoxin to R.F. Apparent linear correlations existed for digoxin and digoxigenin bisdigitoxoside between the concentrations in saliva and plasma measured at identical times after drug administration.

and total polar metabolites can be followed over time intervals sufficiently large for unambiguous delineations of the pharmacokinetics (Figs. 1—4). The recovery, precision and reproducibility performance of methods I, K and L have been characterized and tested in biological samples spiked with standards and/or obtained from pharmacokinetic studies with digoxin. The critical evaluation of the three methods used showed that the assay performance of method L was best. Method L assayed TRA^{bf} and TRP^{bf} accurately and precisely. Methods K and L determined TRA^{bf} adequately; TRP^{bf} assessments by method K were clearly less precise than those by method L. Method I measured DA^{*bf} specifically and precisely. There was evidence that method I estimates of TRP^{bf} (and of TRA^{bf}) were biased. A combination of $(DA^*/TRA)_I^{bf}$ and TRA_L^{bf} obtained, respectively, by methods I and L in the same biological samples gave optimum (unbiased) data which were suitable for pharmacokinetic analysis (Figs. 1—4). It appeared that the anticipated superior assay characteristics of method L were mainly due to the fact that both TRA^{bf} and TRP^{bf} were experimentally measured values with this method, contrary to those with methods I and K.

APPENDIX

Method I

A. Determination of recoveries in biological samples

The recoveries were obtained in experiments where plasma, urine, and saliva samples were spiked with known amounts of DT*, D*, DH_B^*, DB*, (DM+DG+EDG)* and DGL*.

The percentage recovery of DT* with method I, $r_{DT*}^{I,bf}$, was obtained from

$$r_{DT*}^{I,bf} = 10^2 \cdot DT*^{I,bf}/DT_0^{*bf} \quad \text{(A1)}$$

where $DT*^{I,bf}$ represents the measured DT* radioactivity from the scrapings and DT_0^{*bf} the known DT* radioactivity added to a biological sample.

The individual amounts or radioactivities of D_I^{*bf}, DH_I^{*bf}, DB_I^{*bf} and $(DM+DG+EDG)_I^{*bf}$ in a biological sample, DA_I^{*bf}, were obtained from eqn. A2:

$$DA_I^{*bf} = DA^{*I,bf}/r_{DT*}^{I,bf} \quad (A2)$$

where $DA^{*I,bf}$ correspond to the apolar individual radioactivities assignable to D, DH, DB and (DM+DG+EDG) as obtained from the scrapings of the plates from TLC system A. Summation of the individual $DA^{*I,bf}$ values yields $TRA^{I,bf}$:

$$TRA^{I,bf} = \Sigma DA^{*I,bf} \quad (A3)$$

Accordingly the summed individual DA_I^{*bf} values give TRA_I^{*bf}:

$$TRA_I^{bf} = \Sigma DA_I^{*bf} \quad (A4)$$

For the spiked biological samples the percentage recovery of apolar individual radioactivity, $r_{I,DA*}^{bf}$, and the percentage recovery of apolar total radioactivity, $r_{I,TRA}^{bf}$ were obtained from eqns. A5 and A6, respectively

$$r_{I,DA*}^{bf} = 10^2 \cdot DA_I^{*bf}/DA_0^{*bf} \quad (A5)$$

$$r_{I,TRA}^{bf} = 10^2 \cdot TRA_I^{bf}/TRA_0^{bf} \quad (A6)$$

where DA_I^{*bf} and DA_0^{*bf} represent, respectively, measured and known (added) amounts of individual apolar radioactivity and TRA_I^{bf} and TRA_0^{bf} correspond respectively to measured and known (added) apolar total radioactivity.

The percentage recovery of polar total radioactivity (=DGL*), $r_{I,TRP}^{bf}$, for the biological samples tested was obtained from eqn. A7:

$$r_{I,TRP}^{bf} = 10^2 \cdot TRP_I^{bf}/TRP_0^{bf} \quad (A7)$$

where TRP_I^{bf} and TRP_0^{bf} correspond, respectively, to the calculated and known (added) amounts of DGL_0^* radioactivity. The recoveries of DA^{*bf}, TRA^{bf} and of TRP^{bf} obtained by method I are listed in Table III.

B. Determination of unknown concentrations in biological samples

The individual DA_I^{*bf} were obtained by eqn. A8:

$$DA_I^{*bf} = f_I \cdot (10^4 \cdot DA^{*I,bf})/(r_{DT*}^{I,bf} \cdot r_{I,DA*}^{bf}) \quad (A8)$$

where f_I is a correction factor obtained from the experiments which determined the recoveries in samples spiked with the standards (Table III), i.e. $f_I = 10^2/r_{I,DA*}^{bf}$.

TRA_I^{bf} was obtained from eqn. A4 as described above and TRP_I^{bf} was calculated from eqn. A9:

$$TRP_I^{bf} = TR^{bf} - TRA_I^{bf} \quad (A9)$$

Method K

A. Determination of recoveries in biological samples

The recoveries were determined as described above (see method I). The respective percentage recoveries of D* and DGL* in eluate 2 of a biological sample, $r_{D^*}^{K_2,bf}$ and $r_{DGL^*}^{K_2,bf}$, were determined from

$$r_{D^*}^{K_2,bf} = 10^2 \cdot D^{*K_2,bf} / D_0^{*bf} \quad \text{(A10)}$$

$$r_{DGL^*}^{K_2,bf} = 10^2 \cdot DGL^{*K_2,bf} / DGL_0^{*bf} \quad \text{(A11)}$$

where $D^{*K_2,bf}$ and $DGL^{*K_2,bf}$, respectively, are the measured radioactivities of D* and DGL* in eluate 2 of a biological sample and D_0^{*bf} and DGL_0^{*bf} are thus the respective known (added) amounts of D* and DGL* in that biological sample. According to the results obtained with the standards D* and DGL*, the measured radioactivity in eluate 2 represents total radioactivity, $TR^{K_2,bf}$, and eqn. A12 holds:

$$TR^{K_2,bf} = TRP^{K_2,bf} + TRA^{K_2,bf} \quad \text{(A12)}$$

where $TRP^{K_2,bf}$ and $TRA^{K_2,bf}$ correspond, respectively, to polar and apolar total radioactivity in eluate 2 of a biological sample.

Since $TRP^{K_2,bf}$ could be estimated from eqn. A13, assuming that D* and DA* behaved comparably throughout the procedures of method K, i.e. $r_{D^*}^{K_2,bf} \approx r_{DA^*}^{K_2,bf}$, then:

$$TRP^{K_2,bf} = [TR^{K_2,bf} - TR^{bf}\,(r_{D^*}^{K_2,bf}/10^2)]/[1 - (r_{D^*}^{K_2,bf}/r_{DGL^*}^{K_2,bf})] \quad \text{(A13)}$$

The value of TRP_K^{bf} was thus obtained from eqn. A14:

$$TRP_K^{bf} = 10^2 \cdot TRP^{K_2,bf} / r_{DGL^*}^{K_2,bf} \quad \text{(A14)}$$

and TRA^{bf} was calculated subsequently from eqn. A15:

$$TRA_K^{bf} = TR^{bf} - TRP_K^{bf} \quad \text{(A15)}$$

B. Combination of data by methods K and I

Data by methods K and I were combined according to eqn. A16:

$$DA_{I/K}^{*bf} = TRA_K^{bf} \cdot (DA^*/TRA)_I^{bf} \quad \text{(A16)}$$

where TRA_K^{bf} represents apolar total radioactivity as obtained by method K and $(DA^*/TRA)_I^{bf}$ corresponds to the fractional apolar individual radioactivity as determined by method I.

For the biological samples spiked with known amounts of D*, DH_B^*, DB*, DM* and DGL*, the percentage recoveries of polar and apolar total and individual radioactivity, $r_{K,TRP}^{bf}$, $r_{K,TRA}^{bf}$ and $r_{I/K,DA^*}^{bf}$, were thus obtained from eqns. A17—A19:

$$r_{K,TRP}^{bf} = 10^2 \cdot TRP_K^{bf} / TRP_0^{bf} \quad \text{(A17)}$$

$$r^{bf}_{K,TRA} = 10^2 \cdot TRA^{bf}_K / TRA^{bf}_0 \quad \text{(A18)}$$

$$r^{bf}_{I/K,DA^*} = 10^2 \cdot TRA^{bf}_K \cdot (DA^*/TRA)^{bf}_I / DA^{*bf}_0 \quad \text{(A19)}$$

where TRP_K, TRA_K and $DA^*_{I/K}$ correspond, respectively, to the measured amounts of polar total radioactivity, apolar total and individual radioactivity by method K, and TRP_0, TRA_0 and DA^*_0 represent the known (added) amounts of these radioactivities.

The recoveries of TRA^{bf} and TRP^{bf} obtained by method K and the recovery of DA^{*bf} received by combining data from methods I and K are listed in Table IV.

C. Determination of unknown concentrations in biological samples

Unknown TRP^{bf} and TRA^{bf} in biological samples were determined by method K by assaying TR^{bf} and $TR^{K_2,bf}$. The value of TRP^{bf}_K was computed from eqn. A20:

$$TRP^{bf}_K = f_K \cdot (10^2 \cdot TR^{K_2,bf} - TR^{bf} \cdot r^{K_2,bf}_{D^*}) / (r^{K_2,bf}_{DGL^*} - r^{K_2,bf}_{D^*}) \quad \text{(A20)}$$

The correction factor f_K, was obtained from the experiments in which the recoveries in samples spiked with the standards were determined (Table IV), i.e. $f_K = 10^2 / r^{bf}_{K,TRP}$.

TRA^{bf} was calculated from eqn. A15 as indicated previously. Unknown DA^{*bf} was assessed upon combining the results obtained by methods K and I according to eqn. A16 as outlined above.

Method L

A. Determination of recoveries from biological samples

The recoveries were determined as described above (see method I). The measured radioactivities in both the aqueous residue, L_2, and the organic extract, L_1, of a biological sample represented total radioactivity, $TR^{L_2,bf}$ and $TR^{L_1,bf}$, respectively. They were defined by eqns. A21 and A22:

$$TR^{L_2,bf} = TRP^{L_2,bf} + TRA^{L_2,bf} \quad \text{(A21)}$$

$$TR^{L_1,bf} = TRA^{L_1,bf} + TRP^{L_1,bf} \quad \text{(A22)}$$

Since $TRP^{L_2,bf}$ can be estimated from eqn. A23, assuming that D* and DA* behaved comparably throughout the procedures of method L, i.e. $r^{L_2,bf}_{D^*} \approx r^{L_2,bf}_{DA^*}$, then

$$TRP^{L_2,bf} = [TR^{L_2,bf} - TR^{bf} \cdot (r^{L_2,bf}_{D^*}/10^2)] / [1 - (r^{L_2,bf}_{D^*}/r^{L_2,bf}_{DGL^*})] \quad \text{(A23)}$$

$TRA^{L_2,bf}$ can be obtained from eqn. A21 after rearrangement. Similarly, $TRA^{L_1,bf}$ can be estimated from eqn. A24:

$$TRA^{L_1,bf} = [TR^{L_1,bf} - TR^{bf} \cdot (r^{L_1,bf}_{DGL^*}/10^2)] / [1 - (r^{L_1,bf}_{DGL^*}/r^{L_1,bf}_{D^*})] \quad \text{(A24)}$$

$TRP^{L_1,bf}$ was then obtained from eqn. A22 after rearrangement and TR^{bf} was estimated from eqn. A25:

$$TR_L^{bf} = TR^{L_1,bf} + TR^{L_2,bf} \quad \text{(A25)}$$

The ratio of TR^{bf} (measured directly) to TR_L^{bf} (obtained by method L) yields a correction factor q:

$$q = TR^{bf}/TR_L^{bf} \quad \text{(A26)}$$

TRP_L^{bf} and TRA_L^{bf} were obtained, respectively, from eqns. A27 and 28:

$$TRP_L^{bf} = q \cdot 10^2 \cdot (TRP^{L_2,bf}/r_{DGL*}^{L_2,bf}) \quad \text{(A27)}$$

$$TRA_L^{bf} = q \cdot 10^2 (TRA^{L_1,bf}/r_{D*}^{L_1,bf}) \quad \text{(A28)}$$

B. Combination of data by methods L and I

The value of DA^{*bf} could only be assessed if data from methods L and I were combined according to eqn. A29:

$$DA_{I/L}^{*bf} = TRA_L^{bf} \cdot (DA^*/TRA)_I^{bf} \quad \text{(A29)}$$

where TRA_L^{bf} represents apolar total radioactivity as obtained by method L and $(DA^*/TRA)_I^{bf}$ corresponds to the fractional apolar individual radioactivity as determined by method I.

For the biological samples spiked with known amounts of D*, DH_B^*, DB*, DM* and DGL*, the percentage recoveries of polar total radioactivity, apolar total and individual radioactivity, $r_{L,TRP}^{bf}$, $r_{L,TRA}^{bf}$ and $r_{I/L,DA*}^{bf}$, were obtained from eqns. A30—A32:

$$r_{L,TRP}^{bf} = 10^2 \cdot TRP_L^{bf}/TRP_0^{bf} \quad \text{(A30)}$$

$$r_{L,TRA}^{bf} = 10^2 \cdot TRA_L^{bf}/TRA_0^{bf} \quad \text{(A31)}$$

$$r_{I/L,DA*}^{bf} = 10^2 \cdot TRA_L^{bf} \cdot (DA^*/TRA)_I^{bf} \quad \text{(A32)}$$

where TRP_L^{bf}, TRA_L^{bf} and $DA_{I/L}^{*bf}$ correspond to the measured values of these radioactivities as obtained by method L or I and TRP_0^{bf}, TRA_0^{bf} and DA_0^{*bf} represent the known (added) amounts of the standards in the biological samples.

The recoveries of TRA^{bf} and TRP^{bf} obtained by method L and the recovery of DA^{*bf} received by combining data from methods I and L are listed in Table V.

C. Determination of unknown concentrations in biological samples

Unknown TRA^{bf} and TRP^{bf} in biological samples were determined by method L by measuring TR^{bf}, $TR^{L_2,bf}$ and $TR^{L_1,bf}$. TRP^{bf} and TRA^{bf} could be obtained by eqns. A33 and A34, respectively.

$$TRP_L^{bf} = f_{L_2} \cdot q \cdot (10^2 \cdot TR^{L_2,bf} - TR^{bf} \cdot r_{D*}^{L_2,bf})/(r_{DGL*}^{L_2,bf} - r_{D*}^{L_2,bf}) \quad \text{(A33)}$$

$$TRA_L^{bf} = f_{L_1} \cdot q \cdot (10^2 \cdot TR^{L_1,bf} - TR^{bf} \cdot r_{DGL^*}^{L_1,bf}) / (r_{D^*}^{L_1,bf} - r_{DGL^*}^{L_1,bf}) \quad \text{(A34)}$$

where f_{L_2} and f_{L_1} are correction factors obtained from the experiments which determined the recoveries in samples spiked with the standards (Table V), i.e. $f_{L_2} = 10^2 / r_{L,TRP}^{bf}$ and $f_{L_1} = 10^2 / r_{L,TRA}^{bf}$.

LIST OF SYMBOLS AND ABBREVIATIONS

D	= digoxin (unlabelled)
DH	= dihydrodigoxin
DB	= digoxigenin bisdigitoxoside
DM	= digoxigenin monodigitoxoside
DG	= digoxigenin
EDG	= epidigoxigenin
DT	= digitoxin
DGL	= digoxin-16′-glucuronide
D*	= digoxin (labelled)
TR	= total radioactivity (TR=TRA+TRP)
TRA	= apolar total radioactivity
DA*	= apolar individual radioactivity [DA* = D*, DH*, DB*, (DM+DG+EDG)*]
TRP	= polar total radioactivity
DT*, D*, DGL*, DH*, DB*, DM*, TR*, DA*, TRA*, TRP*	= radioactivity, R
R_0^{bf} (p,u,s)	= known added radioactivity in a biological sample (plasma, urine, saliva)
$r_R^{I,bf}$; ($R^{I,bf}$)	= percentage recovery of radioactivity (recovered radioactivity) of a biological sample spiked with DT* as obtained by method I from TLC scrapings
$r_R^{K_2,bf}$, ($R^{K_2,bf}$)	= percentage recovery of radioactivity (recovered radioactivity) of a biological sample spiked with D* or DGL* as obtained by method K in aqueous eluate, K_2
$r_R^{L_2,bf}$, ($R^{L_2,bf}$)	= percentage recovery of radioactivity (recovered radioactivity) of a biological sample spiked with D* or DGL* as obtained by method L in aqueous residue, L_2
$r_R^{L_1,bf}$, ($R^{L_1,bf}$)	= percentage recovery of radioactivity (recovered radioactivity) of a biological sample, spiked with D* or DGL* as obtained by method L in organic extract L_1
$R_{I,K,L}^{bf}$	= measured radioactivity of a biological sample as obtained by methods I, K or L
$R_{I/K,I/L}^{bf}$	= measured radioactivity of a biological sample as obtained by combining data by methods I and K or I and L

$r^{bf}_{I,K,L,R}$ = percentage recovery of radioactivity in a biological sample as obtained by methods I, K or L

$r^{bf}_{I/K,I/L,R}$ = percentage recovery of radioactivity in a biological sample as obtained by combining data by methods I and K or I and L

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