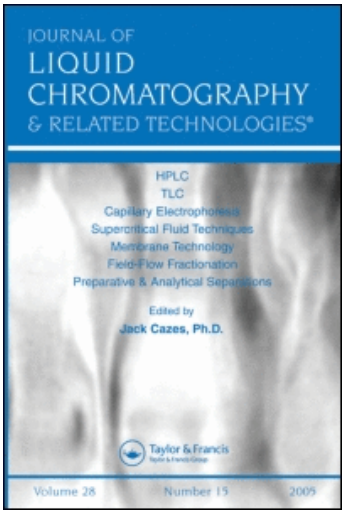

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HPLC Determination of Chloramphenicol, Chloramphenicol Monosuccinate and Chloramphenicol Glucuronide in Biological Matrices

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HPLC DETERMINATION OF CHLORAMPHENICOL, CHLORAMPHENICOL MONOSUCCINATE AND CHLORAMPHENICOL GLUCURONIDE IN BIOLOGICAL MATRICES

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ABSTRACT

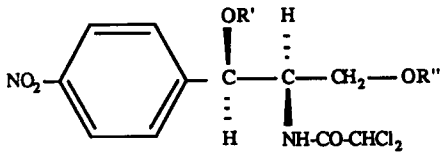
An isocratic reversed-phase liquid chromatographic method for the determination of chloramphenicol 1- and 3-succinate and chloramphenicol in serum within 5 minutes following precipitation with perchloric acid is described. An ion-pair chromatographic method gives the additional option of co-determination of chloramphenicol 3-glucuronide in urine and serum. An extraction method for the determination of chloramphenicol in biological tissues is presented.

INTRODUCTION

The broad spectrum antibiotic chloramphenicol (CAP; MW 323) is used in severe childhood infections of the central nervous system, abscesses caused by anaerobic bacteria and in typhoid fever. Because of its narrow therapeutic concentration range drug level monitoring has been advocated [1,2].

Chloramphenicol is eliminated almost exclusively by metabolism. With the major metabolite being the 3-glucuronide (CAPG) [3], the compound has been used as a model drug in the study of glucuronidation activity *in vitro* as well as *in vivo*.

Chloramphenicol for parentheral use is available as the sodium salt of the monosuccinate ester (CAPS; MW 446). Commercial preparations contain a mixture of the 1- and 3-succinates. Furthermore, the two forms interconvert, the rate being pH-dependent. In aqueous solution the molar fraction of 1-succinate is 0.23 at equilibrium and independent of pH and ionic strength [4].



	R'	R"
Chloramphenicol	H	H
Chloramphenicol 1-succinate	-CO-CH2-CH2-COOH	H
Chloramphenicol 3-succinate	H	-CO-CH2-CH2-COOH

Upon intravenous administration of chloramphenicol succinate, the prodrug hydrolyses to form active chloramphenicol. However, variable amounts of the prodrug, ranging from 13 to 36% of the dose, are recovered in the urine [5]. It is thus fallacious to assume full chloramphenicol bioavailability after intravenous administration of CAPS. For investigative purposes an assay which enables simultaneous determination of chloramphenicol and chloramphenicol 1- and 3-succinate is therefore desired. Previous methods did either not ensure full separation of chloramphenicol 1- and 3-succinate [6,7,8] or required separation times exceeding 10 [9] or 15 minutes [10,11]. A recent routine method for drug monitoring involves acidic extraction to ethyl acetate which is then purified from acidic compounds by extraction with an alkaline aqeuous phase [12]. Although stated that the method may be used for simultaneous determination of chloramphenicol and its succinate esters the procedure appears

unsuitable and in indeed no data was provided as to the determination for the prodrug. Another recent method, being designed for drug monitoring purposes, does not address the analysis of chlorampenicol succinates [13]. A method for metabolic profiling in rat urine utilizing [^{3}H]chloramphenicol has been described [14]. Utilizing a column thermostated at 50°C A previous procedure for co-determination of chloramphenicol and glucuronide, but not monosuccinates, has been described for for the assay of urine samples only [15]. The method, however, required the separation column thermostatted at 50°C. The present paper describes a rapid method for co-determination of chloramphenicol 1- and 3-succinate and chloramphenicol in serum or urine, with an option of direct and simultaneous assay for the glucuronide. Further, a method for determination of chloramphenicol in whole rat blood and various tissues is presented.

MATERIALS and METHODS

Chemicals

Chloramphenicol was obtained from Aldrich (Steinheim, F.R.G) and chloramphenicol sodium monosuccinate from Farmitalia Carlo Erba. In whole blood and tissue assays 3-isobutyl-1-methylxanthine (Aldrich) as used as internal standard. Compound purities were ≥98%. Acetonitrile (Fisons, Loughborough, England) and ethylacetate (Merck, Darmstadt, F.R.G.) were HPLC and LiChrosolv grades respectively, whereas glacial acetic acid (May & Baker, Dagenham, England), sodiumacetate trihydrate and 70% perchloric acid (Merck) were of analytical grade.

Tetrabutylammoniumhydrogenphosphate was obtained from Labkemi AB, Stockholm. Extrelut silica (Merck) was washed with methanol followed by ethanol and dried before use. Stock solutions of bovine liver ß-glucuronidase (Type B-3, Sigma, St. Louis, USA) were prepared by dilution with acetate buffer (pH 5.5) to yield 6000 IE/ml and kept frozen at -20°C in glass test-tubes. D-saccharic acid 1,4-lactone (Sigma, St. Louis, USA), a specific ß-glucuronidase inhibitor, was dissolved in water to yield a 0.24 M stock solution.

Instrumentation

The chromatographic system consisted of a LDC ConstaMetric model III pump, a variable wavelength LDC UV SpectroMonitor III connected to a Hewlett Packard

3396A integrator in the peak area mode, a Rheodyne 7125 injector with a 100 µl loop, and 150-250 mm stainless steel columns (i.d. 4.6 mm) packed with 5µ ODS-Hypersil. Flow rate was 1.5 ml/min. Peaks were monitored at 278 nm.

Mobile phases varied as follows; in the assay for CAPS and CAP in plasma, serum or urine samples 28% acetonitrile in 0.05M (pH 5.5) acetate buffer was used. For the simultaneous determination of CAPS, CAP and chloramphenicol glucuronide in serum and urine, the acetonitrile contents was 25% in 0.05 M (pH 5.9) acetate buffer containing 0.5 mM tetrabutylammonium ion (TBA). Finally, in the determination of CAP in tissues and whole blood, the mobile phase was 28% acetonitrile in a 0.1µ (pH 6.1) phosphate buffer (20 ml 0.5M Na_2HPO_4 + 70 ml 1.0M NaH_2PO_4 diluted to 1 litre). Acetonitrile contents of could be slightly varied depending on what column length was used. Separations were performed isocratically at room temperature.

Assays

Serum: To 100 µl serum or plasma in a polyethylene microtube was added 20 µl 35% perchloric acid and 50 µl methanol. The tubes were placed on a whirlmixer (30 sec) and then centrifuged 5 min (Beckman Microfuge). 50 µl of the clear supernatant was injected on the column.

Blood: 300 µl blood was added to a glass test-tube containing 2.00 ml 50 µM 3-isobutyl-1-methylxanthine (IBMX) internal standard stock solution. After addition of 3.00 ml water, the sample was placed in an ultrasonic bath for 10 minutes, then centrifuged at 40 000g for 15 minutes (4°C). 4.00 ml supernatant was transferred to a 25 cm glass column packed with Extrelut. After 15 minutes, the column was eluted with 20 ml ethyl acetate. The eluate was evaporated under N_2 at 40°C, and reconstituted with 500 µl mobile phase by whirlmixer agitation (30 sec) and ultrasonic bath (10 min) after which 50 µl was injected on the chromatographic column.

Urine: 100 µl urine aliquots were diluted 1:31 with 0.05 M acetate buffer (pH 5.5) to obtain a suitable detector response. 50 µl was injected directly on the column. Of the diluted urine, 1.00 ml was taken into a stoppered glass test-tube and 100 µl ß-glucuronidase solution was added After incubation at 37°C for 24 hrs test-tubes were

centrifuged and 50 µl injected directly on the column. Parallel samples to which also had been added 100 µl saccharolactone to yield a final concentration of 20 mM were run simultaneously.

Tissues: To tissue samples was added 2.00 ml IBMX stock solution and 3.00 ml 0.13 M perchloric acid. After homogenization (10 N Ultra-Turrax) the tubes were treated in the manner described for blood.

Standard curves: Calibration curves were obtained by adding water solutions with known quantities of respective compound to reference matrices. These were: human plasma (Akademiska Hospital blood bank, Uppsala) for human serum analysis, rat plasma for rat plasma analysis, water for urine assays and tissue samples of standardized weights for respective tissue assays. Peak areas were regressed by ordinary least squares against concentration and the solution for the linear function was used for interpolation. Chloramphenicol 1- and 3-succinate peak areas were added to give the total prodrug concentration. In the tissue assay, the peak area ratio of CAP to internal standard was regressed. Calibration curves for chloramphenicol glucuronide in serum were obtained by hydrolyzing an aliquot of an urine sample with ß-glucuronidase, and the molar increase in CAP concentration taken as the quantity of glucuronide in the original sample after correcting for dilution. Aliquots of the same urine sample, chosen for a high CAPG contents and negligible amounts of CAP and CAPS, were then added to plasma.

Serum recoveries were estimated by comparing serum peak areas relative to aqueous solution standards treated in a parallel manner. Estimates for tissue recoveries were obtained by comparing recovered amounts with amounts added to standard tissue samples and correcting for volume transfers;

$$\% \text{ Recovery} = \frac{100 \cdot C_{inj} \cdot V_R \cdot (V_A + W_T) / V_S}{\text{Amount added}}$$

where C_{inj} is the concentration in the final reconstituted sample injected, V_R the reconstitution volume (500 µl mobile phase), V_A the volume added to the original tissue sample (5.00 ml), W_T the weight of the tissue sample and V_S the volume of supernatant transferred post-centrifugation to the Extrelut extraction column (4.00 ml).

RESULTS AND DISCUSSION

The retention of chloramphenicol, being aprotic, was controlled by acetonitrile contents alone. Chloramphenicol monosuccinate (pK_a 4.3) retention was then manipulated by pH. The separation of 1- and 3-succinates and chloramphenicol within 5 minutes in spiked human plasma is shown in Fig 1.

Separation of chloramphenicol, glucuronide and 1- and 3-succinate within 7 minutes was enabled by adjustment of pH, acetonitrile and TBA concentrations. Ion-pair chromatography of a urine sample shows the glucuronide peak (Fig 2a) being virtually absent after treatment with ß-glucuronidase (Fig 2b) but retained upon adding D-saccharic acid 1,4-lactone (Fig 2c).

Accuracy and recovery

The reproducibility, precision and relative recovery of the serum assay are summarized in Table 1. Coefficients of variation (CV) in detector response after

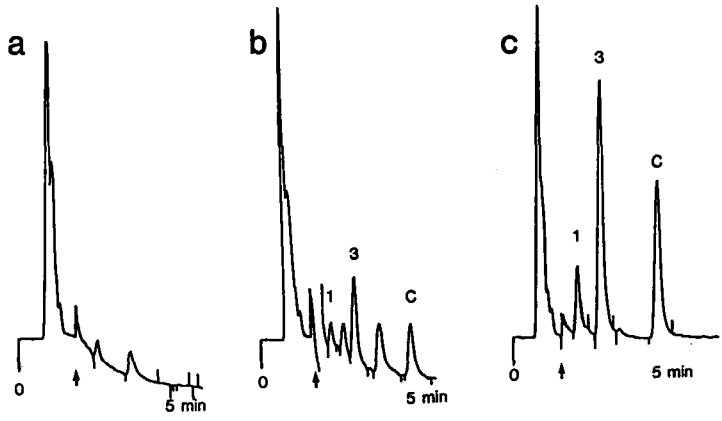


FIGURE 1. Chromatograms of blank human plasma (a), and when spiked with 2 µM CAPS and 1 µM CAP (b) and 50 µM CAPS and 26 µM CAP (c). Peaks 1 and 3 depict chloramphenicol 1- and 3-succinate respectively, C denotes chloramphenicol. Arrows indicate a decrease in integrator attenuation.

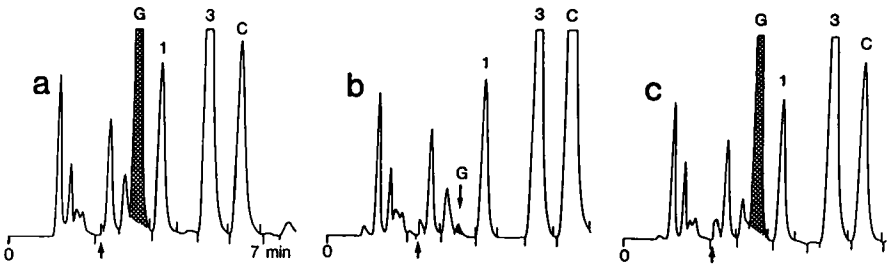


Figure 2: Chromatograms of urine (diluted 1:31) before (a) and after hydrolysis with ß-glucuronidase (b) as well as after parallel treatment with both ß-glucuronidase and D-saccharic acid 1,4-lactone (c). 22 µM chloramphenicol glucuronide (G), 6 µM 1-succinate (1), 21 µM 3-succinate (3) and 10 µM chloramphenicol (C) were separated using the ion-pair mobile phase. The urine sample was obtained 2 hrs after intravenous administration of 77 µmole/kg CAPS to a human subject.

TABLE 1

Serum Assay Intra-day Variability, Accuracy and Recovery (n=10).

		WATER		SERUM		
	Added Conc. (µM)	Determined Conc. (µM)	%CV	Determined Conc. (µM)	%CV	%Recovery
CAPS (1+3)	2.01	1.85	2.0	2.36	3.7	75
	50.2	50.4	1.6	49.7	2.5	79
	251	251	1.7	251	1.9	73
CAP	1.02	0.98	4.0	0.91	4.2	81
	25.5	25.5	1.7	25.6	1.7	84
	102	102	1.2	102	1.2	82

injection of CAPG water solutions utilizing the ion-pair mobile phase were 46, 1.9 and 1.5% for concentrations of 0.3, 6, and 66 μM respectively (n=10). In serum, CVs were 8 and 4% at concentrations of 0.6 and 13 respectively with a recovery of 84%. The assays for serum and urine utilizes no internal standard which necessitates good reproducibility of the volume injected on the column, but does on the other hand not introduce error in volume transfer when adding an internal standard.

Tissue recoveries varied between rat tissues, but were fairly consistent within each type (Table 2). There was a good correlation between mean recoveries of CAP and the internal standard in the different tissues (r = 0.98). Recoveries of both CAP and IBMX declined in a parallel manner with increased tissue sample weight. However, the CAP to IBMX peak area ratio was constant.

Linear detector response CAPS, CAP and IBMX was ascertained in the region 50 pmole to 40 nmole injected on the column as aqueous solutions.

The lower limit of determination is approximately 1μM for all four compounds. Below this concentration, within-run coefficients of variation will rapidly exceed 15%.

TABLE 2.

Recoveries of CAP and IBMX in Tissues (n=8).

	CAP (%)	IBMX (%)
Blood (0.300 μl)	94.1 ± 3.0	99.3 ±2.0
Liver (1.0 g)	52.5 ± 8.8	60.7 ± 1.4
Kidney (0.7 g)	58.8 ± 2.6	69.8 ± 1.1
Lung (0.7 g)	58.0 ± 2.7	67.9 ± 1.8
Heart (0.7 g)	51.3 ± 7.3	66.1 ± 1.1
Gut wall (0.7 g)	70.4 ± 4.6	77.1 ± 3.8
Muscle (1.0 g)	46.9 ± 3.2	60.6 ± 1.8
Brain (1.0 g)	55.6 ± 3.5	65.5 ± 1.5
Spinal cord (0.2 g)	77.3 ± 5.7	89.6 ± 1.9

Stability

Water solutions of CAPS protected from light in lab-bench conditions were stable over a period of approximately two weeks. Spiked human serum samples showed a 50% decrease in CAPS peak areas with a corresponding increase for CAP over a period of two weeks, whether protected from light or not. The addition of 20 µl 35% perchloric acid to a 100 µl sample increased CAPS rate of hydrolysis in both water and human serum with a 50% decrease in peak area after 45 and 80 hrs respectively, with a concomitant increase in chloramphenicol peaks. To counteract hydrolysis during sample workup, no more than three samples were therefore handled at the same time whenever using perchloric acid for protein precipitation. Water solutions of chloramphenicol glucuronide were stable for at least three hours after addition of perchloric acid. Thus glucuronide levels in serum are not expected to interfere with the assay by degradation during workup.

The half-life of CAPS following incubation in fresh human blood (37°C) was 20 hrs. In thawed blood bank serum the corresponding half-life was 38 hrs. These slow rates of hydrolysis confirm earlier findings that blood esterases are not an important site of CAPS hydrolysis in humans [16,17].

The preferred method for cleaving the conjugate was by enzymatic hydrolysis. CAPS and CAP in water solution were both stable when subjected to ß-glucuronidase. Although chloramphenicol glucuronide was unstable under alkaline conditions (pH≥12) with a half-life of 17 minutes, there was no corresponding increase in aglycone levels. Chloramphenicol is subjected to hydrolysis of covalent-bound chlorine in alkaline solutions [18].

Perchloric acid precipitation proved to be the superior method for the tissue assay. Trichloroacetic acid gave a yellow coloring when added to a water solution of CAP and IBMX (48 hrs; 6°C) with recoveries of 93 and 80% respectively. Enzymatic tissue digestion (2 mg Subtilisin, 55°C, 1 hr in pH 7.5 Tris buffer) was faulted due to on-going metabolism during work-up.

Applications

Mean serum concentrations after a single intravenous dose of 100 mg/kg chloramphenicol sodium succinate to Sprague-Dawley rats (200 g) are shown in Fig. 3.

Following a single intravenous dose of 25 mg chloramphenicol per kg bodyweight (as chloramphenicol sodium succinate) serum from six subjects was assayed with the ion-pairing mobile phase, enabling simultaneous determination of chloramphenicol glucuronide (Figure 4). The terminal slope of the glucuronide approaches that of CAP suggesting the probability of formation-limited metabolite kinetics.

Fifty-seven urine samples collected from 33 patients on chloramphenicol therapy was assayed before and after treatment with ß-glucuronidase. An excellent correlation was found between chloramphenicol glucuronide peak area and increase in chloramphenicol concentration in a parallel sample after enzymatic hydrolysis (Fig 5). When the decrease in glucuronide peak areas was regressed against the increase in chloramphenicol peak areas a slope factor of 1.061±0.009 was obtained, suggesting that the glucuronide has similar UV absorptive properties as chloramphenicol on a

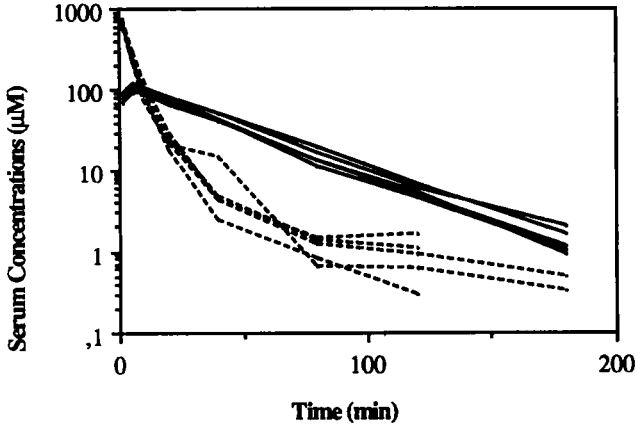


FIGURE 3: Serum concentrations of CAPS (dashed lines) and CAP (unbroken lines) in 5 rats after intravenous injection of 225 µmole/kg chloramphenicol succinate. The median chloramphenicol half-life was 26 min.

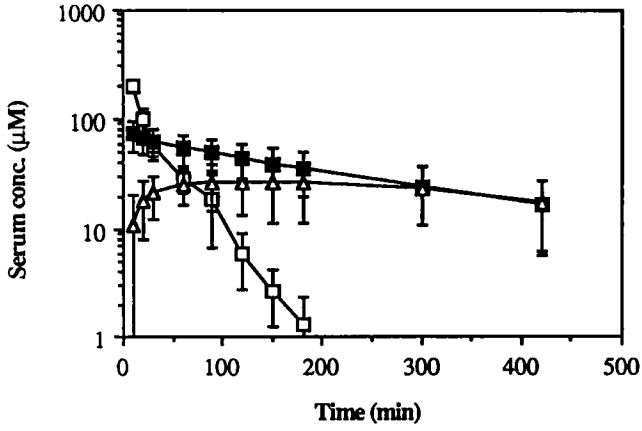


FIGURE 4: Mean serum concentrations (±SD) of 1+3 CAPS (□), CAP (■) and CAPG (△) in six human subjects following intravenous dose of 77 µmole/kg chloramphenicol sodium succinate.

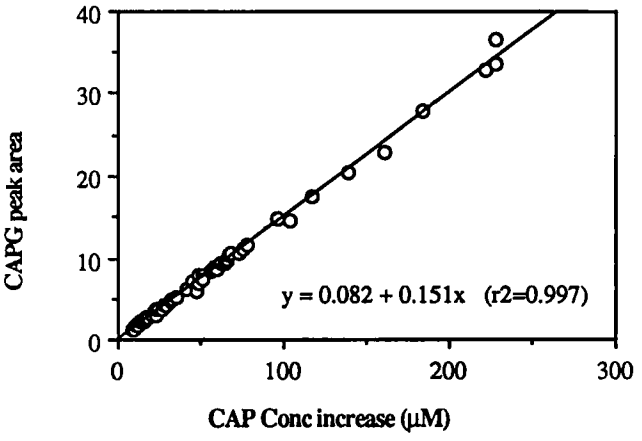


FIGURE 5: Correlation between chloramphenicol glucuronide peak areas prior to, and increase in chloramphenicol concentration following, ß-glucuronidase hydrolysis. 57 urine samples (diluted 1:31) collected after intravenous dose of 77 µmoles chloramphenicol sodium succinate were assayed by ion-pair chromatography.

molar to molar basis at the wavelength used. Hydrolysis was complete since residual peaks with the same retention time as CAPG were, after ß-glucuronidase treatment, only 2.9 ± 2.3% (n=57) of the original peak area. The same samples run with saccharolactone added retained 100.3 ± 5.4% of original glucuronide peak areas, indicating total inhibition and constituting evidence for the specificity of the hydrolysis. The 3-monoglucuronide has been identified as the major chloramphenicol metabolite but the existence of other glucuronides was not excluded [2]. Assuming that a 1-glucuronide or a 1-and 3-diglucuronide are unlikely to co-elute with the 3-monoglucuronide, the excellent correlation between glucuronide peak areas and the increase in CAP concentrations after ß-glucuronidase in the present study suggests that only the 3-glucuronide is formed in humans.

Male 210g Sprague-Dawley rats were sacrificed at 5, 15, 40, 90, 160 and 250 min after a tail vein injection of 75 mg CAP/kg. After decapitation, tissues were cleaned and freed of blood on an ice-cold dissection plate. A standardized weight of each tissue was added to a polyethylene centrifuge tube and immediately stored at -25 °C until analysis. Plasma, blood and tissues were assayed as described. Fig 6 shows the correlation between plasma concentrations and, for brevity, those of blood (r^2=0.98), gut wall

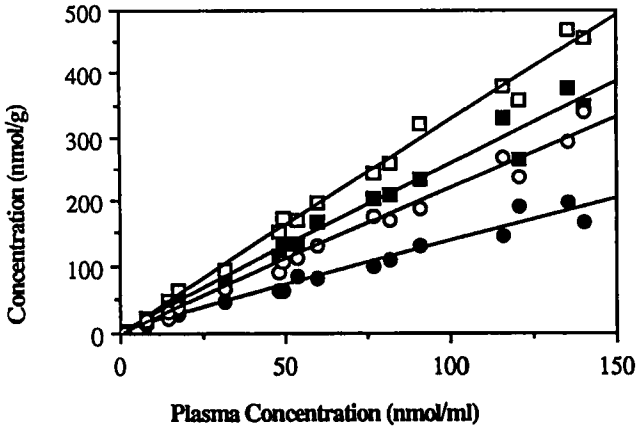


FIGURE 6: Relationship between chloramphenicol plasma concentrations and concentrations in blood (●), gut (○), lungs (■) and kidneys (□) respectively, following an intravenous dose of 230 µmole/kg in rats.

(r^2=0.98), lung (r^2=0.98) and kidney (r^2=0.99). Of these well-perfused organs the highest concentrations, relative to plasma, were found in kidneys, followed by lungs and gut wall.

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