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Biopharmaceutical Studies of Thiazide Diuretics. II.[1] High-Performance Liquid Chromatographic Method for Determination of Hydrochlorothiazide in Plasma, Urine, Blood Cells and Bile

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A rapid, specific and sensitive high-performance liquid chromatographic method for the determination of hydrochlorothiazide in small volumes of plasma, urine, blood cell and bile was developed. The method has been applied in a drug level study in patients given a single oral dose of 100 mg of hydrochlorothiazide. The drug was extracted into ethyl acetate from biological fluid, and then the endogenous substances were eliminated with ion exchange cellulose and silica gel. A methanol solution of the drug extract, containing hydroflumethiazide as an internal standard, was chromatographed on a silica gel column with a mobile phase containing *n*-hexane and ethanol. Eluted components were detected by ultraviolet absorption measurement at 270 nm. Based on 0.3 ml of plasma, the limit of detection of the method was 5 ng/ml, and the detector response was linear in the range of 20—1600-ng/ml with an overall recovery of 96.8 ± 1.5%. Similarly, detector responses were linear over the concentration ranges of 5 to 200 μg/ml for urine, 20 to 400 ng/ml for hemolysate, and 20 to 600 ng/ml for bile. No interference was observed in this assay from the following drugs which may be administered concurrently with hydrochlorothiazide therapy: prednisolone, spironolactone, deslanoside, nifedipine, penicillins and cephalosporins.

Keywords——hydrochlorothiazide; high-performance liquid chromatography; patient receiving diuretic therapy; plasma concentration; urinary concentration

Hydrochlorothiazide (HCT) has been clinically used for a number of years, and several analytical procedures have been reported for determining this diuretic in biological specimens. Colorimetric methods[2] used to measure HCT in urine are based on derivatization involving the Bratton–Marshall reaction, and do not possess adequate sensitivity and specificity. Gas-liquid chromatographic methods[3] are specific, but require extensive sample clean-up and time-consuming derivatization.

High-performance liquid chromatographic (HPLC) methods were recently introduced for HCT assay because of their high specificity and sensitivity. However, it is difficult to determine plasma concentration of diuretics of patients with renal disease, because their blood contains many endogenous substances. Cooper[4a] failed due to contamination problems in an attempt to increase the sensitivity. The method of Christophersen[4b] requires gel filtration to remove plasma components, but again the limit of detection was 50 ng/ml. Robinson's method[4c] rquired 5 ml of plasma, and Barbhaiya's approach[4a] required 10 ml of blood. Although we reexamined their methods, we could not obtain satisfactory results.

A new method for the determination of HCT in human plasma, urine, blood cell and bile by using HPLC is described here. The method is simple, rapid, precise and sensitive, and

requires only 0.3 ml of plasma of patients receiving therapeutic doses of this drug. Beerman[5] reported that more than 95% of absorbed HCT was excreted unchanged. Therefore, in analyzing the fluids, no consideration was given to metabolites.

Experimental

Materials——Powder and tablets (Esidrex®) of HCT were obtained from Ciba-Geigy (Japan) Ltd. Silica gel (Wakogel C-200, Wako Pure Chemical Industries) and diethylaminoethyl cellulose (Whatman DE52, Whatman Ltd.) were used as adsorbents for impurities. Solvents were of chromatographic grade. Other reagents were of special grade (Wako Pure Chemical Industries), and were used as obtained.

Apparatus——The HPLC instrument used in this study was a TRIROTAR-II chromatograph (Japan Spectroscopic Co.) equipped with a UVIDEC-100-III spectrophotometric detector (Japan Spectroscopic Co.) set at 270 nm. The column (250 × 4.6 mm, i.d.) was packed with 5-μm diameter silica gel (Fine SIL-5, Japan Spectroscopic Co.), and a precolumn (23 × 3.8 mm, i.d.) of porous silica gel (Perisorb A, Merck) was fitted to protect the main column from plasma or urinary components. The flow rate of the mobile phase was adjusted to 2.0 ml/min and the column pressure was 70—80 kg/cm^2. All chromatograms were recorded at a chart speed of 2.5 mm/min. Operations were carried out at room temperature. Concentrations were determined by the method of peak height ratios.

Mobile Phase——Mobile phase A (for plasma) was 18% (v/v) ethanol and 7% (v/v) dichloroethane in *n*-hexane. Mobile phase B (for urine) was 22% (v/v) ethanol in *n*-hexane. Mobile phase C (for hemolysate and bile) was 15% (v/v) ethanol and 10% (v/v) dichloroethane in *n*-hexane. Each mobile solvent was degassed with an ultrasonic apparatus and filtered through a stainless steel porous disc (Japan Spectroscopic Co.) before use.

Standard Solution——Since a preliminary study indicated that HCT was unstable in alkaline media for prolonged periods, stock solutions of HCT were prepared by dissolving HCT in methanol to give a concentration of 100 μg/ml and then diluting this with methanol to give solutions of 10, 5, 2 μg/ml. The solutions were stable when stored at −20 °C for one month or more.

Plasma: Standard solutions (10—100 μl) were placed in 15-ml glass-stoppered centrifuge tubes, and evaporated to dryness at room temperature under reduced pressure with a rotary evapo-mixer (Vapour Mix, Tokyo Rika). A mixture of 0.3 ml of blank plasma and 1.0 ml of purified water was added to each tube and mixed for 5 min to cover the concentration range between 0.02 and 0.6 μg/ml of plasma.

Urine: A mixture of 10 μl of blank urine and 990 μl of purified water was added to the dry residue of HCT to prepare a working standard in the range of 5—150 μg/ml as described above for plasma.

Blood Cells: Blank blood (0.4 ml) was centrifuged to separate plasma and blood cells. The plasma layer was decanted off, and the blood cells were hemolyzed by the addition of 1.0 ml of purified water, and diluted ten times with purified water. Then 3.0 ml of hemolysate was added to the dry drug residue to prepare a working standard in the range of 0.02—0.4 μg/ml.

Bile: A mixture of 0.3 ml of blank bile and 2.7 ml of purified water was added to the dry residue of HCT to prepared working standards in the range of 0.02—0.3 μg/ml.

Internal Standard Solution——A hydroflumethiazide (HFM) (Sankyo Co.) solution in methanol was used as the internal standard. It was stable throughout the experiments.

Extraction——Plasma: A plasma sample (0.3 ml) was diluted with 1.0 ml of purified water, and mixed with about 60 mg of diethylaminoethyl cellulose. Then 8.0 ml of ethyl acetate was added, and the mixture was shaken mechanically for 10 min, and centrifuged. In order to remove persistent impurities, 7.0 ml of the organic layer was transferred to another tube and about 50 mg of silica gel was added. The content was shaken for 5 min and centrifuged. The ethyl acetate layer (6.0 ml) was transferred to a tube and evaporated to dryness at room temperature. The residue was reconstituted in 30 μl of methanol containing 2.5 μg of HFM/ml as the internal standard, and 10 μl was injected into the chromatograph.

Urine: A mixture of 10 μl of urine and 990 μl of purified water was extracted with 7.0 ml of ethyl acetate. The organic layer (6.0 ml) was transferred to a tube, evaporated to dryness, and reconstituted in 30 μl of methanol containing 20 μg of HFM/ml as the internal standard; 6 μl was injected.

Hemolysate: Heparinized blood was transferred to several ($N=6-8$) 50-μl hematocrit tubes, and centrifuged at 11000 rpm (13000 × g) for 5 min, then the hemotocrit value (H_{CT}) was measured. The tubes were cut at the plasma-blood cell interface, put into a centrifuge tube, and 1.0 ml of purified water was added. The centrifuge tube was vigorously stirred, exposed to ultrasonic treatment to cause hemolysis and centrifuged. The hemolysate (0.3 ml) was mixed with 2.7 ml of purified water. The mixture was washed with 8 ml of chloroform. Two and a half milliliters of the water phase was transferred to another tube, and mixed with 60 mg of diethylaminoethyl cellulose and 8.0 ml of ethyl acetate. The ethyl acetate layer was removed and evaporated as described above for plasma. The residue was reconstituted in 30 μl of methanol containing 1.25 μg of HFM/ml as the internal standard, and 20 μl was injected. The concentration in the blood cells (C_R) was calculated by using the hematocrit value:

$$(50 \times N \times H_{CT}/100 + 1000) \times C_D = (50 \times N \times H_{CT}/100) \times C_R \quad (1)$$

$$C_R = (1 - 2000/N \times H_{CT}) \times C_D \quad (2)$$

where C_D is the concentration in hemolysate.

Bile: A bile sample (0.3 ml) was mixed with 2.7 ml of purified water. The mixture was washed with 8 ml of chloroform and extracted with ethyl acetate as described above for hemolysate.

Clinical Study——Single doses of 100 mg of HCT (as four tablets of Esidrex®, 25 mg) were administered to fifteen healthy volunteers and thirty-one patients. All doses were administered at 9 a.m.; the tablets were swallowed whole with 100 ml of water. A light breakfast was served 2 h before the drug administration. Blood samples (1 ml) were taken from the forearm vein into a heparinized syringe, and centrifuged immediately to separate the plasma and the blood cells. Urinary samples were collected quantitatively through 24 or 48 h postdosing. Bile was collected from a catheter inserted into the bile duct of a patient with choledocholithiasis. All samples were stored frozen at −20 °C until assayed.

Partition Coefficients——Partition coefficients of HCT between water and various organic solvents were measured following the method described previously.[1)]

Results and Discussion

Extraction

Table I shows the true partition coefficients of HCT between the buffer (pH 5.0) and several organic solvents. Although ketones seem to be good extracting solvents, they were not satisfactory because they extract many endogeneous substances. Ethyl acetate was selected as a more selective solvent. Robinson[4c)] reported that the use of ethyl acetate for the extraction of HCT from serum resulted in emulsion formation. However, no emulsion with ethyl acetate was obtained by our method, and the recovery was good.

The pK_a of HCT is 8.78,[1)] and its partition coefficient is maximum in the pH range of 2—7.[1)] In our experiment, the extraction into ethyl acetate was not facilitated by dilution of the plasma with phosphate or acetate buffer.

It was not necessary to remove plasma protein for extraction. A single extraction procedure is enough for the isolation of HCT.

Clean-Up Procedure

The most important process in the determination of the plasma concentration of diuretics is the clean-up procedure. Several procedures for the removal of endogeneous substances which are the cause of interfering peaks were investigated. Preextraction with organic solvents such as ether,[4a)] toluene [4d)] and methylene chloride[8)] were all unsatisfactory

TABLE I. True Partition Coefficients of Hydrochlorothiazide

Organic solvent	Partition coefficient[a)]
Methyl isobutyl ketone	28.9
Methyl ethyl ketone	11.3
Ethyl acetate	10.4
n-Octanol	0.97
Ethyl ether : dichloromethane : 2-propanol (3 : 2 : 1)[b)]	0.96
Ethyl ether : dichloroethane (1 : 1)[c)]	0.57
Chloroform	0.022
Carbon tetrachloride	Less than 0.01
Benzene	Less than 0.01

a) At 25±2 °C.
b) Reported in ref. 6.
c) Reported in ref. 7.

due to the removal of plasma components; besides, repeated extraction and preextraction result is scattering of the measured values. Gel filtration[4b] is expensive and time-consuming.

Addition of ion exchange cellulose and silica gel proved to be ideally suited for the removal of persistent impurities in the biological samples. Almost complete clean-up was obtained with between 30 and 150 mg of ion exchange cellulose, and between 20 and 100 mg of silica gel.

Lipoid-rich fluid, such as hemolysate and bile, requires preextraction with an organic solvent. We found that chloroform was more effective than the other solvents tested: toluene, hexane and dichloromethane.

Chromatography

The HPLC determination of HCT[4a,b] was performed by reversed-phase liquid partition chromatography. Although this method is ideally suited for determinations of nonpolar drugs, it is difficult to separate such drugs from lipoids. Ion exchange chromatography was not sensitive, and showed tailing. Since we obtained increased efficiency by using adsorption chromatography, we chose to chromatograph HCT on silica gel. With hexane containing ethanol as the mobile phase, good efficiency was achieved. The retention time for HCT in plasma was found to be 12 min with no interfering peaks, and it decreased as the percentage of ethanol increased. The aging of packing materials shortened the retention time.

HCT has an ultraviolet absorption maximum at 226 nm,[4a,c] but this wavelength was found to be unsuitable for quantitative analyses since interfering peaks were observed. Another maximum exists at 270 nm[1] and, though it is less intense, this was used for routine assay.

Representative chromatograms of plasma, urine, blood cells and bile samples from patients are shown in Fig. 1. Two unknown compounds from the blank bile shown in Fig. 1 could not be removed by preextraction with the organic solvents. Columns prepared at different times gave reproducible void volumes and results. The following drugs, which may be administered concurrently with HCT therapy, did not interfere with the assay: pre-

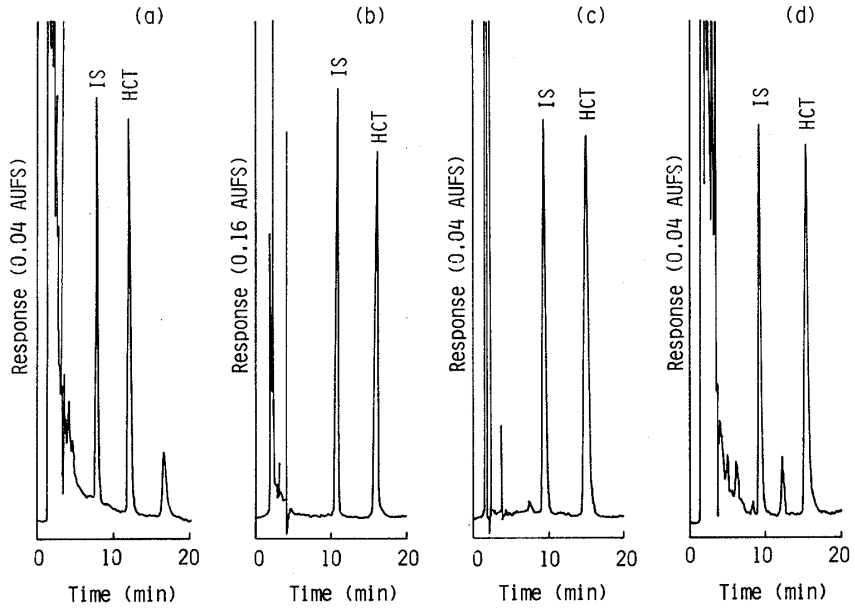

Fig. 1. Chromatograms of Hydrochlorothiazide from Patients' Body Fluids

IS, internal standard (hydroflumethiazide); HCT, hydrochlorothiazide; (a), plasma of patient 19 containing 0.4 μg of HCT/ml; (b) urine of patient 8 containing 80 μg of HCT/ml; (c), hemolysate of patient 10 containing 0.4 μg of HCT/ml; (d), bile of patient 11 containing 0.3 μg of HCT/ml.

donisone, levodopa, spironolactone, deslanoside, nifedipine, dipyridamole, penicillins and cephalosporins.

Calibration Graph

The peak height ratios (drug/internal standard) were plotted against drug concentration in μg/ml of fluids. The calibration graph was constructed for the concentration range of 0.02—0.6 μg/ml plasma, with the attenuator set at 0.04 AUFS (Fig. 2). For higher concentrations (0.6—1.2 μg/ml) present in plasma, an attenuation of 0.08 AUFS was sufficient. The detector response was linear in the 0.02—1.6-μg/ml range.

Figure 2 also shows other calibration graphs obtained by HPLC assay. There was a good linear relationship between the peak height ratio and the HCT concentration. When a high concentration was expected, calibration graphs were prepared with large amounts of internal standard added to standard solutions and samples.

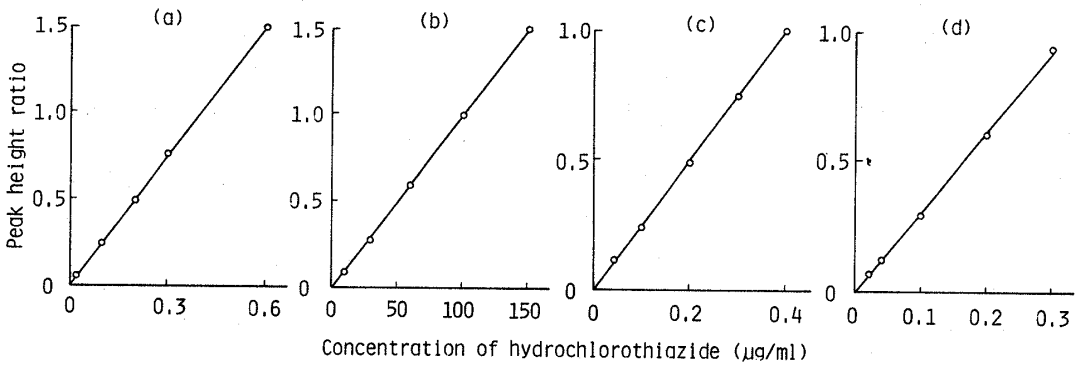

Fig. 2. Typical Calibration Graphs of Hydrochlorothiazide from Plasma, Urine, Hemolysate and Bile

(a), plasma; (b), urine; (c), hemolysate; (d), bile.

TABLE II. Recovery of Hydrochlorothiazide from Plasma, Urine, Hemolysate and Bile

Fluid	Concentration of hydrochlorothiazide (μg/ml)	Recovery (%)
Plasma	0.2	97.5±2.1[a]
	0.6	96.8±1.6
	1.0	96.6±1.6
	1.6	96.5±0.9
Urine	20	93.9±1.1
	70	95.5±1.2
	140	97.2±0.8
Hemolysate	0.2	78.8±2.8
	0.4	77.6±2.3
	0.8	77.2±1.4
Bile	0.1	83.0±1.5
	0.2	80.9±1.5
	0.4	81.9±1.4

a) Each value represents the mean±S.D. of four determinations.

Recovery and Sensitivity

The recovery of added HCT from blank fluids was independent of concentration (Table II). In the cases of plasma and urine, satisfactory recoveries were obtained. A double ethyl acetate extraction did not give higher HCT recovery. While the recovery was reproducible from day to day, we prefered to prepare a calibration graph for each set of analyses.

A recovery of $77.9 \pm 2.2\%$ (mean $\pm$ S.D., $n=12$) was obtained when HCT (0.2—0.8 μg/ml) was added to hemolysate. The reason why the recovery is incomplete was probably the partial interaction of HCT with biochemical constituents. Similar binding might occur in the bile.

Based on the response obtained with 0.3 ml of plasma, the detection limit of the method was assessed as 5 ng/ml (4 mm peak; about 4 times the baseline noise) when 20-μl portions of reconstituted solution were injected. Christophersen[4b] reported a detection limit of 50 ng/ml using 1 ml of serum, and Robinson *et al.*[4c] obtained a detection limit of 2 ng/ml using 5 ml of serum in their HPLC assay.

Application of the Method

The availability of the method for preclinical and clinical purposes was studied. The concentration of HCT in the body fluids of fifteen healthy volunteers given HCT was measured. Interference by endogenous substances was not a major problem, and clear chromatograms were obtained. A typical experiment is shown in Fig. 3.

Fluids from thirty-one patients receiving HCT therapy were assayed. All of the results were calculated from calibration graphs obtained by adding HCT to blank fluid from patients. Twenty-five of these patients concomitantly received other drugs. None of these drugs caused interference with the determination of HCT. A typical result is shown in Fig. 4. However, the chromatograms for patients 25 and 31 showed an unknown peak which was not separated completely from the HCT peak (Table III). Three of eighty samples (from 15 healthy volunteers and 31 patients) were disturbed by small interfering peaks. The cause of the interfering peak has not yet been elucidated.

The method described here is sufficiently sensitive to detect plasma concentrations of HCT for 48 h and to measure urinary excretion for 48—72 h after an oral dose of 100 mg HCT. The procedure for plasma has marked advantages over previous methods[3] in both

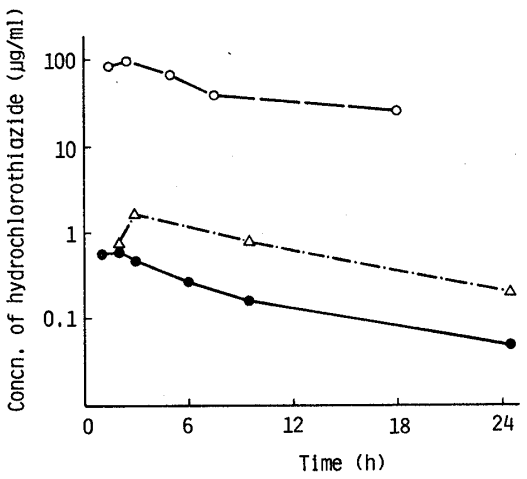

Fig. 3. Semilogarithmic Plots of Concentration of Hydrochlorothiazide in Samples from a Healthy Subject (Male, 26 Years, 57 kg) after a 100-mg Oral Dose

●, plasma; ○, urine; △, blood cells.

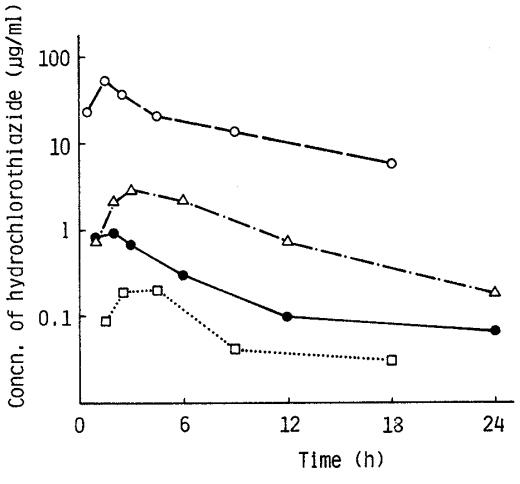

Fig. 4. Semilogarithmic Plots of Concentration of Hydrochlorothiazide in Samples from Patient 11 after a 100-mg Oral Dose

●, plasma; ○, urine; △, blood cells; □, bile.

TABLE III. Patients Receiving Hydrochlorothiazide Therapy

Patient	Sex	Age (years)	Weight (kg)	Disease	Sample[a]	Other medicaments taken
1	M	53	48	Nephrotic syndrome	P, U	Chondroitin sulfate, ascorbic acid
2	F	73	43	Chronic pyelonephritis	P, U	Aspirin, nitrazepam, sulpiride, nalidixic acid, sulpiride, sennoside A & B
3	M	66	55	Myocardial infarction	P, U	Metildigoxin, coenzyme Q, diazepam, dilazep
4	F	45	43	Idiopathic edema	P, U	None
5	F	58	45	Lung cancer	P, U	Nifedipine, colloidal aluminium hydroxide, ascorbic acid
6	M	34	57	Hypertension	P, U	None
7	F	27	45	Chronic glomerulonephritis	P, U	None
8	F	21	49	Nephrotic syndrome	P, U	Prednisolone, dicyclomine, cefalexin
9	M	48	55	Diabetic nephropathy	P, U	Propranolol, hydralagine, dipyridamole, allopurinol
10	M	69	59	Nephrotic syndrome	P, U, C	Pancreatin, dipyridamole, phenovalin, pirenzepine
11	F	57	47	Choledocholithiasis	P, U, C, B	Cocarboxylase, flavin adenine dinucleotide, vitamin K2, cefazolin
12	M	76	52	Chronic renal failure	P	Phenovalin, domperidone, lactulose, sorbitol
13	M	58	48	Liver cirrhosis	P	Ursodesoxycholic acid, estazolam, pantethine
14	M	73	55	Lung cancer	P	Apricot kernel, tipepidine hibenzoate
15	F	69	37	Hypothyroidism	P	Metochlopramide, bromhexyne, dried thyroid, gentian
16	M	31	68	Chronic renal failure	P	Aluminium hydroxide gel
17	F	12	42	Chronic renal failure	P	Nicardipine, propranolol, hydralazine, thiamine
18	F	76	44	Idiopathic thrombocytopenia	P	Levodopa, ε-aminocapronic acid, mepirizole, flavoxate
19	M	61	60	Chronic glomerulonephritis	P	Dipyridamole, estazolam, dextran sulfate sodium
20	M	23	54	Nephrotic syndrome	U	Dicyclomine, diazepam, gentian, prednisolone
21	M	32	72	Chronic glomerulonephritis	U	None
22	M	55	44	Nephrotic syndrome	U	Cyclophosphamide, clotiazepam, hydrotalcite, prednisolone
23	M	72	50	Chronic renal failure	P, U	Furosemide, nifedipine, propranolol, hydralazine
24	F	49	58	Diabetes mellitus	U	Lactomin, sennoside A & B
25	F	63	50	Diabetes mellitus	U†	Serratio peptidase, bromhexyne, lactomin
26	M	35	54	Diabetes mellitus	U	Insulin
27	M	63	51	Diabetes mellitus	U	Trapidil, adenosine triphosphate, acetohexamide
28	M	25	62	Acute hepatitis	U	None
29	M	47	57	Peptic ulcer	U	Basic aluminum sucrose sulfate, iron sorbital, dicyclomine
30	M	31	84	Hypertension	U	None
31	M	70	49	Gastric cancer	P,† U, C,† B	Carmofur, mepenzolate, doxycycline, pantethine

a) P, plasma; U, urine; C, blood cell; B, bile. †, interfering peak.

sensitivity and specificity. The procedures are different for plasma, urine and hemolysate, but the use of identical extraction and chromatographic conditions for HCT in the fluids makes the methods simple, accurate, and convenient for pharmacokinetic studies.

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