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SIMULTANEOUS ANALYSIS OF DIGITOXIN AND ITS CLINICALLY RELEVANT METABOLITES USING HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND RADIOIMMUNOASSAY

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SUMMARY

A specific assay for determing the urinary excretion of unchanged digitoxin and its metabolites is described. The procedure includes solvent extraction of urine at pH 8.5, reversed-phase high-performance liquid chromatography (HPLC) and radioimmunoassay (RIA) of equivalent fractions. Confidence limits showed good linearity and precision of recovery and high sensitivity, accuracy and specificity. Cross-reactivities were high for digitoxigenin (DGTN) and digitoxigenin bisdigitoxoside (Bis-DGTN), they were low for digitoxigenin monodigitoxoside (Mono-DGTN) or digoxin when [^{125}I]digitoxin RIA was used. The interference of endogenous compounds in urine in the RIA was overcome by using HPLC. Compared with results reported in the literature, the urinary recovery of unchanged digitoxin was lower, being only $8.11 \pm 1.51\%$ of the dose administered. Amounts of $6.52 \pm 1.31\%$ were excreted hydrolysed as Bis-DGTN, Mono-DGTN, DGTN or C_{12}-hydroxylated as digoxin.

INTRODUCTION

Therapy with currently available cardiac glycosides is often complicated by adverse reactions [1,2]. Digoxin (DG) is predominantly eliminated by the kidneys [3], whereas digitoxin (DGT) is mainly biotransformed in the liver [4,5]. Radioimmunoassay (RIA) [5,6] and red blood cell uptake [7] have been widely used for measuring plasma concentrations and urinary excretion of DGT. These methods are sensitive for the determination of DGT concentrations in biological fluids but do not distinguish unchanged DGT from its metabolites. Therefore, more specific techniques have been developed, including double isotope dilution derivative assay [8], ^{86}Rb uptake after thin-layer chromatography (TLC) [9], gas chromatography (GC) [10,11], high-performance liquid chromatography (HPLC) [12,13], TLC methods [14–16] and column chromatography [17,18].

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The double isotope dilution derivative assay using an HPLC method is generally not very sensitive for the measurement of DGT and its metabolites in serum or urine. In contrast, GC is very sensitive, but the need for several steps of sample clean-up and derivatization makes it time-consuming. RIA after TLC using a normal- or reversed-phase system to separate the parent drug from its metabolites does not provide information about the interference of DGT metabolites with endogenous compounds in urine [19–21]. Recently, a method combining HPLC and RIA has been reported for the quantitation of DG, DGT and their metabolites in human tissues [22]. However, the occurrence of endogenous material in urine which may interfere with the DGT assay has not been previously investigated.

The aim of this study was the development of a specific and accurate method in which DGT and its major metabolites could be simultaneously determined in urine. The procedure for the separation of DGT from its major urinary metabolites uses a reversed-phase HPLC system after a simple clean-up, which is followed by a radioimmunoassay of the isolated compounds.

EXPERIMENTAL

Subjects

Eight healthy volunteers [mean age 28 4 ± 2.6 years, body weight 70.5 ± 2.3 kg; mean ± standard error of the mean (S.E.M.)] without cardiovascular, renal, hepatic or endocrinal disease were studied. Each subject received an oral dose of 0.5 mg of DGT in the morning and an identical dose of DGT 12 h afterwards. Urine was collected at the following times: 0–4, 4–8, 8–12, 12–24 h, and then at 24-h intervals up to 168 h.

Materials

The following unlabelled compounds were purchased from Sigma (St. Louis, MO, U.S.A.) and Serva (Heidelberg, F.R.G.): DG, digitoxigenin (DGTN), DGT, digitoxigenin monodigitoxoside (Mono-DGTN) and digitoxigenin bisdigitoxoside (Bis-DGTN). [^{3}H]DGT (13.8 Ci/mmol) and [^{3}H]DG (13.8 Ci/mmol) were obtained from New England Nuclear (Boston, MA, U.S.A.) and purified prior to use with HPLC. Hydrolysed labelled metabolites were obtained by hydrolysis of pure [^{3}H]DGT after incubation with 1 *M* hydrochloric acid at room temperature for 20 min. The reaction mixture was then neutralized with an equal volume of 1 *M* sodium hydroxide solution and extracted three times with 5 ml of methylene dichloride. The organic solvent was evaporated to dryness under a gentle stream of nitrogen at 37 °C and the residue dissolved in 0.15 ml of methanol.

Separation of the hydrolysed compounds was performed by HPLC as described below and eluted fractions were collected every minute. The radioactivity of a small aliquot of each was measured by liquid scintillation spectrometry in 10 ml of a scintillation mixture (Quickszing I; ZINSSER-Analytical, U.K.). Retention volumes of the tritium-labelled compounds were monitored and then compared with a chromatogram of the unlabelled metabolites.

Extraction procedure

[^{3}H]DGT was diluted about 100–1000-fold with phosphate-buffered saline merthiolate (PBSM) phosphate buffer (pH 7.0) containing 0.1% of bovine serum albumin, 0.9% of sodium chloride and 0.1% of thimerosal added to an equal volume of distilled water (Ampuwa; Fresenius, Bad Homburg, F.R.G.). Approximately 5500 cpm of chromatographically pure [^{3}H]DGT was added as internal standard to 1 ml of urine in a 150×15 mm glass screw-capped tube and vortexed. The same amount of labelled DGT was added to a scintillation vial containing 0.45 ml of mobile phase to equalize any quenching of samples and 9.5 ml of scintillation liquid for the monitoring of the recovery. A 0.2-ml volume of 1 *M* sodium hydroxide solution (E. Merck, Darmstadt, F.R.G.) was added, the sample was vortexed, extracted at pH 8.5 for 2 min with 5 ml of methylene dichloride, centrifuged at 1700 *g* for 15 min and the aqueous phase aspirated. The organic layer was washed with 1 ml for 0.1 *M* sodium hydroxide solution for 20 s and the aqueous layer was aspirated and centrifuged at 1700 *g* for 10 min. Each tube containing the organic layer plus residual aqueous solution was immersed in a container of liquid nitrogen for 10 s and then the organic phase was decanted into a tube. The methylene dichloride was evaporated and the residue was dissolved in 0.15 ml of methanol by vigorous vortexing and then analysed by HPLC.

High-performance liquid chromatography

HPLC was performed on a system consisting of a Spectra-Physics SP 8700 liquid chromatograph solvent-delivery system connected to a Kratos variable-wavelength UV detector operating at 222 nm and to Gilson 201 fraction collector (Villiers le Bel, France) with a 201-202 controller. A Chromatopac C-R 3A computing integrator (Shimadzu, Kyoto, Japan) was used to record the chromatograms. All injections were performed manually every 38 min using a Rheodyne 7125 injector (Rheodyne, Berkeley, CA, U.S.A.) (100-μl loop) and nineteen fractions every 2 min were collected. Separation of DGT and its metabolites was performed in a reversed-phase column using a non-linear gradient and automatic mixture of methanol (solvent A) and water containing PIC B7 reagent (solvent B) (Waters Assoc., Milford, MA, U.S.A.). The initial proportion of methanol (66%) was increased at 0.4% per min for 5 min and by 0.58% per min for the next 12 min. The gradient elution was then followed by isocratic elution with methanol–water (75:25) for 8 min. The last peak was eluted with a retention time of 21 min and the system was re-equilibrated with 66% methanol in water for a further 13 min prior to the next injection. A 25 cm×4 mm I.D. column was filled with Nucleosil RP-5 C_{18} (5 μm) spherical silica gel (Macherey-Nagel, Düren, F.R.G.).

Radioimmunoassay

For the assay of Mono-DGTN, DGTN, Bis-DGTN and DGT, aliquots of 0.5 ml of the HPLC eluates from the appropriate fraction 3, 5, 9, 10 or 11 were transferred to coat a count [^{125}I]DGT RIA kit (DPC, Los Angeles, CA, U.S.A.) and evaporated to dryness. Blank serum (0.05 ml) was added to the sample tube followed by [^{125}I]DGT in buffer (1 ml from a kit). The tubes were vortexed for

15 s, then incubated for 1 h at 37°C. The contents were aspirated and the tubes were assayed for radioactivity in a gamma counter (Kontron MR 480) for 1 min. For the assay of DG, 0.2-ml aliquots of eluates from the appropriate fractions 4 were transferred to a coated tube [^{125}I]DG RIA kit (DPC) and treated as described for DGT, replacing [^{125}I]DGT in buffer with [^{125}I]DG in buffer from the kit.

A calibration graph using a spiked sample of blank urine was prepared for each compound. Blank urine samples were subjected to extraction, HPLC was performed and fractions were collected corresponding to the retention times of each compound. Aliquots of the appropriate fractions were transferred to coated tubes and evaporated to dryness. Standards in serum were provided in the kits for DG (0.025–0.400 ng per assay) or DGT (0.25–4.00 ng per assay). For the remaining metabolites standards were prepared in blank serum at the same concentrations as for DGT. To all standards [^{125}I]DGT or [^{125}I]DG in buffer was added and assayed as described. The calibration graph for each compound was automatically plotted as logit % bound (^{125}I) against the amount (ng per tube). Sample amounts were calculated automatically from the calibration graphs.

The interference of urinary endogenous compounds with the RIA was investigated after adding 0.5–20.0 ng of a mixture containing the five compounds to 1 ml of blank urine. This was followed by extraction and separation by HPLC and RIA in an aliquot of appropriate fractions Mono-DGTN (NR3), DG (NR4), DGTN (NR5), Bis-DGTN (NR9) and DGT (NR10-11). The specificity of this procedure was confirmed by adding 2.0 ng of a mixture containing the five compounds to 1 ml of blank urine. This was followed by extraction, HPLC separation and RIA as described above.

Recovery monitoring

The recoveries of DGT and its metabolites were studied after adding 0.1–10.0 ng of each labelled compound to 1 ml of urine. The recoveries of unlabelled DGT and its metabolites were investigated after adding 0.5 ng of DG and 1.0–10.0 ng in a mixture of the four other unlabelled compounds. The same amount of internal standard, equivalent to 0.1 ng of [^{3}H]DGT per tube, was added to 1 ml of blank urine. The amounts of internal standard added were subtracted when calculating the initial DGT concentration in standards and samples.

An aliquot of 0.5 ml of eluate (fractions 10+11) from HPLC was mixed with 9.5 ml of scintillant and the radioactive content determined in a liquid scintillation counter (UNISOLV-1). The recovery was calculated using the equation

$$\text{Recovery (\%)} = \frac{\text{counts in sample}}{\text{total counts}} \cdot \frac{V_r V_E \cdot 100}{V_i V_a}$$

where V_r represents the volume of methanol added to reconstitute a dried sample (150 μl), V_i the volume injected into the HPLC system (100 μl), V_E the volume of eluate from HPLC for DGT (3.2 μl) and V_a the volume of the aliquot added to a vial (0.5 μl).

The confidence limit of the method was based on the linearity of the RIA and HPLC techniques, the sensitivity of the RIA and HPLC procedures and the

reproducibility determined as the coefficient of variation and calculated after ten repeated measurements on individual samples (concentration range 0.1–20.0 mg/ml of urine). Between-day and within-day variations were monitored over one week by repeated determinations of DGT and its metabolites in urine. The accuracy was calculated as the percentage fractional differences between observed and actual concentrations.

Data analysis

Statistical analysis was performed by a Hewlett-Packard 9848B computer. Urinary excretion was expressed as percentage of the administered oral dose (mean ± S.E.M.). Linear regression analysis was performed using the method of least squares.

RESULTS AND DISCUSSION

Extraction, chromatography and monitoring of recovery

The extraction of DGT and its metabolites from urine with methylene dichloride was found to be most efficient at pH 8.5, with recoveries of 100% of Mono-DGTN and DGTN, 95.3% of DGT, 91% of DG and 88% of Bis-DGTN. Following extraction, DGT and its metabolites were separated by reversed-phase HPLC using a two-step gradient. Fig. 1 compares the separations of metabolites and DGT achieved with an isocratic or gradient system.

The molar absorptivities of DGT and its metabolites at the wavelengths used (220 or 254 nm) prevented direct photometric detection in plasma at a therapeutic level of 9–30 ng/ml. On measuring at 220 nm instead of 254 nm, the sensitivity for DG and its metabolites was increased 10–20 fold for DG and Mono-DGTN and 10–50 fold for DGT, Bis-DGTN and DGTN in this study, as already observed previously [21]. As therapeutic concentrations of DGT and of its metabolites cannot be detected photometrically, another method must be used to determine the retention volumes of all compounds in the samples. This problem was solved by monitoring the added ^{3}H-labelled metabolites by liquid scintillation spectrometry of the eluate.

Fig. 2 shows a typical chromatogram of urine to which 0.1 ng/ml ^{3}H-labelled stand rd mixture was added. The peaks observed at 220 nm represent solvent and endogenous material from urine. Aliquots of the appropriate fractions from the column were analysed quantitatively by RIA to investigate the interference of endogenous compounds in the urine. In order to avoid contamination of the HPLC system with relatively large amounts of DGT and its metabolites, the retention times of standards were monitored at daily intervals by UV spectrometry at the end of the analytical procedure. The interference of endogenous compounds is more pronounced in the assays with Mono-DGTN and DG than in those with DGTN, DGT or Bis-DGTN, as shown in Fig. 3.

Appropriate fractions of extracted urine samples injected into the HPLC system were collected and analysed by liquid scintillation spectrometry. The recovery of each ^{3}H-labelled compound was determined from the ratio of the amount of the labelled compound added to that eluted from the column.

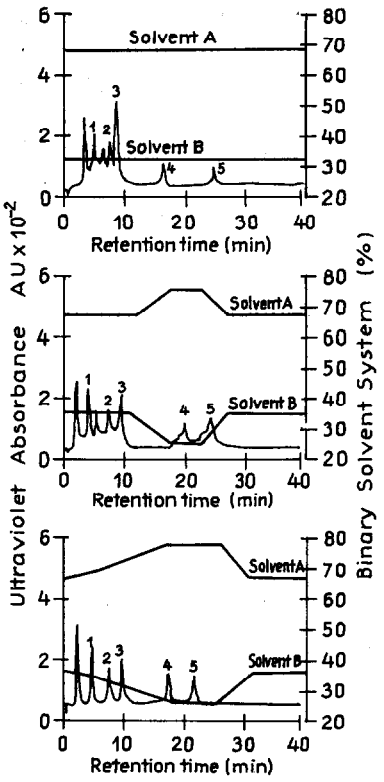

Fig. 1. HPLC of submicrogram amounts of DGT and its metabolites (0.5 μg of each) using a reversed-phase system. Nucleosil C_{18} (5 μm) column (25 cm × 4.6 mm I.D.); mobile phase containing methanol (solvent A) and water plus PIC B7 (solvent B); flow-rate, 0.8 ml/min; pressure 115 bar; isocratic separation (top), one-step gradient (middle) and two-step gradient (bottom). Peaks: 1 = Mono-DGTN; 2 = DG; 3 = DGTN; 4 = Bis-DGTN; 5 = DGT.

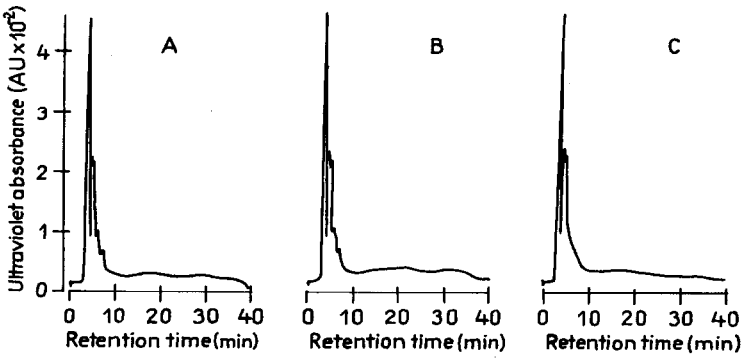

Fig. 2. HPLC of a human urine extract using a two-step gradient of subnanogram amounts of [^{3}H]DGT and labelled metabolites (0.1 ng of each per ml urine). Peaks: solvent and endogenous compounds extracted from human urine samples. (A) Urine blank; (B) mixture of standards extracted from urine; (C) urine sample from a volunteer.

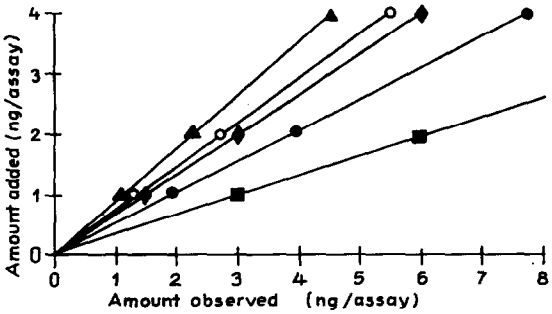

Fig. 3. Interference of endogenous material in urine in RIA after extraction and chromatography. Compounds: (■) Mono-DGTN; (○) Bis-DGTN; (●) DGTN; (▲) DG; (♦) DGT.

As described previously [21] for DG and its hydrolysed metabolites, these unlabelled metabolites were prepared after acidic hydrolysis, extraction and purification by HPLC. The recoveries were found to be 70% after 10 min and 100% after 20 min of incubation for Mono-DGTN, Bis-DGTN and DGTN. Complete dissolution of the residue of evaporated HPLC fractions was obtained after adding 0.05 ml of serum plus 1 ml of [^{125}I]DGT or [^{125}I]DG in buffer. Monitoring of the recovery of the unlabelled compounds was performed by RIA after adding amounts of 0.5 ng/ml of a mixture of all the compounds in urine.

Quantitation

RIA was performed on the samples following HPLC with standards prepared from plasma. The background activity caused by interfering urinary compounds was determined after extraction and HPLC of blank extracts (Fig. 3). In spite of high interference in the first fractions eluted by HPLC, the analytical interference of the endogenous compounds in RIA was eliminated. The standards of DGT and its metabolites in serum were added to tubes containing the evaporated appropriate fraction of blank extract. Utilizing this procedure, the background activity caused by endogenous material in urine was eliminated.

The cross-reaction with DGT and with the DG antibody was studied for the four metabolites of DGT. The results are presented in Table I. Owing to the high percentage of cross-reaction it was possible to quantitate DGTN, Mono-DGTN

TABLE I

SPECIFICITY OF COAT A COUNT [^{125}I]DGT RIA PROCEDURE FOR DGT METABOLITES AFTER EXTRACTION AND CHROMATOGRAPHY

Compound	Amount added (ng/ml)	Apparent DGT concentration (mean ± S.E.M.) (μg/ml)	Cross reaction (mean ± S.E.M.) (%)
DG	2.00	0.46 ± 0.02	23 ± 1
DGTN	2.00	1.56 ± 0.03	78 ± 2
Mono-DGTN	2.00	0.39 ± 0.01	20 ± 1
Bis-DGTN	2.00	1.82 ± 0.05	91 ± 3

TABLE II

CALIBRATION GRAPH USING URINE, INTERCEPT, SLOPE AND CORRELATION COEFFICIENT FOR THE LOG-LOGIT CALIBRATION GRAPH CALCULATED AUTOMATICALLY BY THE GAMMA COUNTER FOR COMBINED HPLC-RIA PROCEDURE

Compound	Range (ng/ml)	Intercept	Slope	Linear correlation coefficient*
DGT	5-80	−0.01167	−1.30704	0.99402
Bis-DGTN	5-80	−0.61437	−0.65870	0.97570
Mono-DGTN	5-80	0.66925	−0.74810	0.90008
DGTN	5-80	−0.34383	−0.88106	0.98621
DG	0.5-8.0	−0.61188	−0.85696	0.98382

*$y = ax + b$.

and Bis-DGTN using [^{125}I]DGT RIA, but [^{125}I]DG RIA was preferred for the quantitation of DG. Concentrations of metabolites in urine samples were corrected automatically for the recovery, blank and added metabolites using calibration graphs generated in an automatic gamma counter.

The confidence limits of the analytical methods are shown in Tables II and III. In this study, the combination of HPLC with RIA improved the sensitivity 10–100 fold compared with the performance of the HPLC method alone. Using RIA with less specific separating techniques, such as TLC [12,15,18–20], variable values for the excretion of DGT and its metabolites were obtained. However, a cross-reactivity of the digitoxin antibody used was not reported for all metabolites and

TABLE III

CONFIDENCE LIMITS OF ANALYTICAL METHOD FOR DETERMINATION OF DGT AND ITS METABOLITES IN URINE

Method	Parameter	DGT	Bis-DGTN	Mono-DGTN	DGTN	DG
HPLC	Linearity (μg/ml)	0.5-10.0	0.5-10.0	1.0-20.0	0.5-10.0	1.0-20.0
	Sensitivity (μg per assay)	0.3	0.3	0.8	0.3	0.8
	Mean Recovery ($n=8$) (range 0.1-10 μg/ml) (%)	100	100	100	100	100
	Mean accuracy ($n=5$) (%)	3.2	2.5	3.5	2.8	1.4
	Mean precision ($n=10$): within-day	1.2	1.5	1.8	2.0	2.5
	between-day	3.5	5.0	4.5	3.8	5.0
HPLC + RIA	Linearity (ng/ml)	1-120	1-120	4-200	1-120	0.1-15.0
	Sensitivity (ng per assay)	0.05	0.05	0.2	0.05	0.005
	Mean recovery ($n=8$) (range 0.1-10 ng/ml) (%)	95	88	100	100	91
	Mean accuracy ($n=5$) (%)	1.3	3.5	3.7	2.5	1.2
	Mean precision ($n=10$): within-day	3.2	2.8	4.5	3.5	4.8
	between-day	4.8	3.9	6.7	5.0	7.2

TABLE IV

URINARY EXCRETION OF DIGITOXIN AND METABOLITES IN HEALTHY VOLUNTEERS

Compound	Amount excreted (mean ± S.E.M., $n=8$) (% of given dose)
DGT	8.11 ± 1.15
Bis-DGTN	3.40 ± 0.70
Mono-DGTN	0.22 ± 0.07
DGTN	1.18 ± 0.32
DG	1.72 ± 0.71

the interference of endogenous material in urine with the RIA has so far not been investigated.

Human studies

The amounts of DGT and the metabolites excreted in urine as percentage of the dose are listed in Table IV. Utilizing the method described, unchanged DGT is excreted in smaller amounts than previously reported [20]. Only 8.11 ± 1.51% of the dose administered was excreted as unchanged DGT, while 14.62 ± 2.26% was excreted as DGT plus metabolites. Amounts of 6.52 ± 1.31% were excreted hydrolysed as Bis-DGTN, Mono-DGTN and DGTN or after C_{12}-hydroxylation as digoxin.

REFERENCES

1 G.C. Oliver, B.M. Parker, D.L. Brasfield and C.W. Parker, J. Clin. Invest., 47 (1968) 1035.
2 K. Rasmussen, J. Jervell and O. Storstein, Eur. J. Pharmacol., 3 (1971) 236.
3 E.E. Ohnhaus, P. Spring and L. Dettli, Dtsch. Med. Wochenschr., 99 (1974) 1797.
4 L. Storstein, Clin. Pharmacol. Ther., 16 (1974) 25.
5 W. Kirch, E.E. Ohnhaus, P. Dylewicz, J. Pabst and L. Storstein, Am. Heart J., 111 (1986) 325.
6 T.W. Smith, J. Pharmacol. Exp. Ther., 175 (1970) 352.
7 J.M. Lowenstein and E.M. Corril, J. Lab. Clin. Med., 67 (1966) 1048.
8 D.S. Lukas and R.E. Peterson, J. Clin. Invest., 45 (1966) 782.
9 L. Storstein, J. Chromatogr., 117 (1976) 87.
10 E. Wilson, S.A. Johnson, W.H. Perkins and J.E. Ripley, Anal. Chem., 39 (1967) 40.
11 J.W. Meilink, J.B. Lenstra and A. Baerheim-Svendsen, J. Chromatogr., 170 (1979) 35.
12 W. Lindner and R.W. Frei, J. Chromatogr., 117 (1976) 81.
13 F.J. Evans, J. Chromatogr., 88 (1974) 411.
14 G. Züllich, W. Braun and B.P. Lisboa, J. Chromatogr., 103 (1975) 396.
15 R.T. MacFarland, F.I. Markus, P.E. Fenster, P.E. Graves and D. Perrier, Eur. J. Clin. Pharmacol., 27 (1984) 85.
16 H.F. Vöhringer, N. Rietbrock, P. Spurny, J. Kuhlmann, H. Hampl and R. Baethke, Clin. Pharmacol. Ther., 19 (1976) 387.
17 G.T. Okita, F.E. Kelsey, P.J. Talso, L.B. Smith and E.M.K. Geiling, Circulation, 7 (1953) 161.
18 G. Züllich, K.H. Damm and B.P. Lisboa, J. Chromatogr., 115 (1975) 117.
19 P.E. Graves, P.E. Fenster, R.T. MacFarland, F.I. Markus and D. Perrier, Clin. Pharmacol. Ther., 36 (1974) 601.

20 P.E. Graves, P.E. Fenster, R.T. MacFarland, F.I. Markus and D. Perrier, Clin. Pharmacol. Ther., 36 (1974) 607.
21 H.A. Nelson, S.V. Lucas and T.P. Gibson, J. Chromatogr., 163 (1979) 169.
22 J. Plum and T. Daldrup, J. Chromatogr., 377 (1986) 221.