Journal of Chromatography, 382 (1986) 167—174
Biomedical Applications
Elsevier Science Publishers B.V., Amsterdam — Printed in The Netherlands

CHROMBIO. 3292

DETERMINATION OF BENZARONE IN HUMAN PLASMA AND URINE BY HIGH-PERFORMANCE LIQUID CHROMATOGRAPHY AND GAS CHROMATOGRAPHY—MASS SPECTROMETRY

IDENTIFICATION OF THE CONJUGATES

JAN X. DE VRIES*, I. WALTER-SACK, A. VAN DE LOO and J. KOCHER

Medizinische Universitätsklinik, Abteilung Klinische Pharmakologie, Bergheimer Strasse 58, D-6900 Heidelberg 1 (F.R.G.)

(First received April 22nd, 1986; revised manuscript received June 23rd, 1986)

SUMMARY

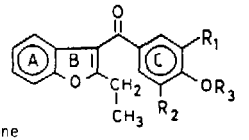
Benzarone (the debrominated metabolite of the uricosuric drug benzbromarone) has been proposed for treatment of vascular disorders. An assay was developed for the quantitation of total benzarone (conjugated and unconjugated) in plasma and urine, following oral intake of benzarone. Enzymatic hydrolysis of the samples with β-glucuronidase/arylsulphatase, extraction, gradient elution high-performance liquid chromatography with reversed-phase columns and UV detection were used for the assay. The concentration ranges, precision and sensitivities were: 0.01—2 μg/ml, 3—5% and 0.01 μg/ml, respectively, for both plasma and urine. These results were validated by gas chromatography—mass spectrometry after methylated derivatives were prepared. Enzymatic hydrolysis of plasma with pure β-glucuronidase or arylsulphatase showed that the relative amounts of unconjugated, glucuronidated, and sulphated benzarone were 6, 12 and 82% respectively, for both plasma and urine.

INTRODUCTION

Benzbromarone (Fig. 1) is the main uricosuric drug used for treatment of gout [1]. Its debrominated metabolite benzarone (2-ethyl-3-benzofuranyl 4-hydroxyphenyl methanone) (Fig. 1) [1—3] is also pharmacologically active [4]. Although it is weakly uricosuric it has not been used to treat gout [1]; it has been shown to have fibrinolytic activity and is proposed for treatment of vascular disorders [4—6].

Several studies on the simultaneous assay of benzbromarone and benzarone

0378-4347/86/$03.50



	R_1	R_2	R_3
1. Benzarone	H	H	H
2. Bromobenzarone	Br	H	H
3. Benzbromarone	Br	Br	H
4. Benzarone-O-CH_3	H	H	CH_3
5. Benzarone conjugate (O-sulphate)	H	H	$-SO_3^-$
6. Benzarone conjugate (O-glucuronide)	H	H	β-D-glucuronic acid
7. Bromobenzarone-O-CH_3	H	Br	CH_3
8. Benzbromarone-O-CH_3	Br	Br	CH_3
9. Benzarone-O-C^2H_3	H	H	C^2H_3
10. Bromobenzarone-O-C^2H_3	Br	H	C^2H_3
11. Benzbromarone-O-C^2H_3	Br	Br	C^2H_3

Fig. 1. Structures of benzarone and derivatives.

following application of the parent drug have been published using methods based on radioactivity [2], gas chromatography (GC) [7], gas chromatography—mass spectrometry (GC—MS) [8] and high-performance liquid chromatography (HPLC) [9]. Few publications have reported on drug levels when benzarone itself was given as a drug [10, 11]; in addition to the above-mentioned assays, a radioactive [10] and an HPLC technique [11] have been published.

HPLC methods were reevaluated and found not to be selective enough; therefore a new HPLC assay was developed for the measurement of plasma concentrations and urinary excretion of benzarone in humans following oral administration of benzarone; the results obtained with this method will be reported in a separate paper [12]. Plasma and urine were also analysed for free (unconjugated) drug and conjugated metabolites, as no data about their structure and relative amounts have been published.

EXPERIMENTAL

Apparatus

Liquid chromatography was carried out with a system composed of two pumps (Models 510 and 6000A), a WISP Model B automatic injector, a Model 660 solvent programmer (all from Waters-Millipore, Eschborn, F.R.G.), a Model SPD-2A variable-wavelength UV detector and a Model C-R1B printer plotter (Chromatopac, Shimadzu, Düsseldorf, F.R.G.). GC—MS analysis was performed with a Model 5986A quadrupole system (Hewlett-Packard, Bad Homburg, F.R.G.).

Reagents

All test substances were analytical-reagent grade. Benzarone, bromobenzarone and benzbromarone (Fig. 1) were generous gifts from Dr. W. Stüber (Zentrallaboratorium Deutscher Apotheker, Eschborn, F.R.G.); warfarin was purchased from Sigma (Taufkirchen, F.R.G.); solvents for liquid chromatography were spectroscopic grade (Merck, Darmstadt, F.R.G.) and for GC—MS Nanograde quality (Promochem, Wesel, F.R.G.). Enzymes for hydrolysis were β-glucuronidase/arylsulphatase from *Helix pomatia* (12 and 60 U/ml) (Merck),

β-glucuronidase from *Escherichia coli* (20 U/ml) and arylsulphatase from *H. pomatia* (25 U/ml) (Boehringer Mannheim, Mannheim, F.R.G.); enzymatic activities and specificities were measured using 4-nitrophenyl-β-glucuronide or sulphate as substrate [13].

Plasma and urine samples

Eight healthy normal male volunteers (24—34 years) received a single oral dose of benzarone (100 mg) (as pellets in hard gelatine capsules). Venous blood was collected in heparinized tubes at various time intervals and centrifuged. The plasma was kept at −23° C. Urine was collected during 24 h, cooled and stored at the same temperature. Samples were thawed and centrifuged before analysis. The clinical design and results will be reported elsewhere [12].

Extractions

(A) Total benzarone in plasma and urine. In a 15-ml centrifuge tube, plasma (1 ml), acetate buffer (100 μl; 1 *M*; pH 4.5) and β-glucuronidase/arylsulphatase (10 μl) were incubated at 37° C for 20 h [14]; warfarin (internal standard, 5 μg), hydrochloric acid (200 μl, 3 *M*) and 1,2-dichloroethane (6 ml) were added, and the stoppered tubes shaken for 10 min horizontally at room temperature and centrifuged at 800 *g* for 10 min. The lower phase (5 ml) was transferred to another clean tube, and the solvent was evaporated under a nitrogen stream in a water-bath at 50° C. Before HPLC analysis the residue was dissolved into 100 μl of solvent A, and 20 μl were injected. Urine samples were hydrolysed and extracted in the same way as described above for plasma.

(B) Enzymatic hydrolysis of plasma and urine with pure β-glucuronidase or arylsulphatase. To determine the ratio of benzarone glucuronide to sulphate, 1 ml of plasma (or urine) was incubated as above with the same amount of pure β-glucuronidase or arylsulphatase; samples were extracted following various periods of incubation (2, 4, 8 and 22 h) and analysed as above.

(C) Non-conjugated benzarone in plasma and urine. No enzyme was added before extraction and analysis.

(D) GC—MS analysis. For the micropreparative isolation of benzarone, plasma or urine extracts were injected several times into the HPLC system and the peak was collected at the retention time of 7.4 min. The solvent was evaporated and the residue was methylated as previously described [15, 16]. The residue was dissolved in 10 μl of isooctane, and 1 μl was injected into the GC—MS system in the electron-impact (EI) and scanning mode.

For the quantitation and validation of the HPLC method, extracts were methylated [15, 16], and analysed in the GC—MS system in the EI and selected-ion monitoring (SIM) mode at *m/e* values 249, 265, 279, 280, and 322.

Chromatographic conditions

HPLC. Gradient elution control settings were: initial and final conditions, 20% and 100% B, respectively; gradient programme run, 30 min; convex gradient No. 5 ($y = 20x^{0.5}$; y = gradient in % B, x = time in min) with a total flow-rate of 2.0 ml/min was used; solvents A and B were mixtures of acetonitrile—water—acetic acid (300:700:5 for A, and 800:200:5 for B). Each run

lasted 8 min for analysis and 10 min for reequilibration to initial conditions. A reversed-phase C_{18} column was employed [LiChrosorb RP-18 with 10-μm irregular particles; 250 × 4 mm I.D. Hibar RT 250-4 (Merck); room temperature]. A guard column (30 × 4 mm I.D.) was packed with Bondapak C_{18} Corasil (35—50 μm spherical particles, Waters). The system pressure was 7—14 MPa. After a series of analyses the column was washed with water and methanol. Peaks were detected at 313 nm with a sensitivity of 0.01 a.u.f.s.

Gas chromatography—mass spectrometry. GC and MS system parameter settings and conditions were as previously described [15, 16].

Standard solutions and calibrations

Benzarone and warfarin standard solutions (10 mg in 100 ml of methanol) were stable for several weeks at 4°C in the dark; 5 μg of warfarin and benzarone (0.05, 0.1, 0.2 and 0.5 μg) were added to each 1 ml of pretreatment plasma or urine, and extracted as described in extraction A. The calibration was calculated from the regression curve (ratio of the peak area of benzarone to that of the internal standard versus added benzarone concentration in μg/ml).

RESULTS

Plasma

Fig. 2a shows the chromatogram of an extract from pretreatment plasma. The Fig. 2b curve shown was obtained from a volunteer 1.5 h after benzarone intake, the plasma having been treated with β-glucuronidase/arylsulphatase before extraction (extraction method A). Fig. 2c was derived from plasma without enzymatic treatment (extraction method C). Plasma samples were hydrolysed separately with pure β-glucuronidase and arylsulphatase (method B). Benzarone by definition means total amounts of drug, including the part that originates from conjugated metabolites.

Only small amounts of benzarone appear in the non-conjugated form (Fig. 2c; Fig. 3 curve I); incubations with pure β-glucuronidase and pure arylsul-

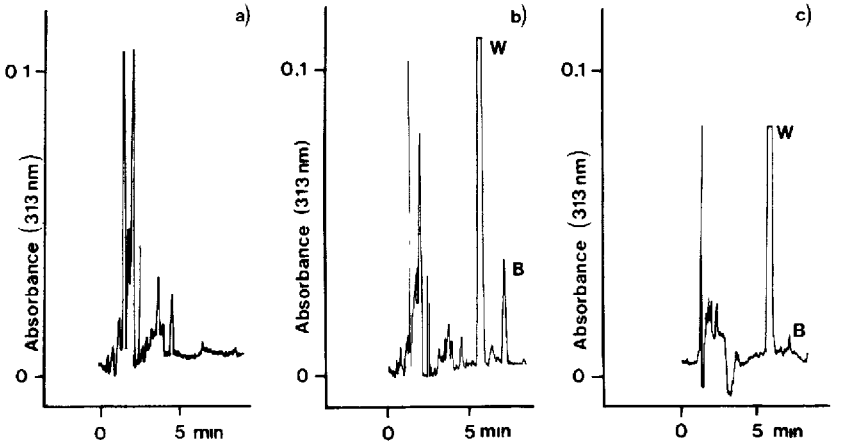


Fig. 2. HPLC of extracts from volunteer's plasma. (a) Pretreatment plasma, (b) plasma collected 2 h after benzarone application and following β-glucuronidase/arylsulphatase hydrolysis; (c) as in (b) but without hydrolysis. Peaks: W = warfarin (internal standard); B = benzarone.

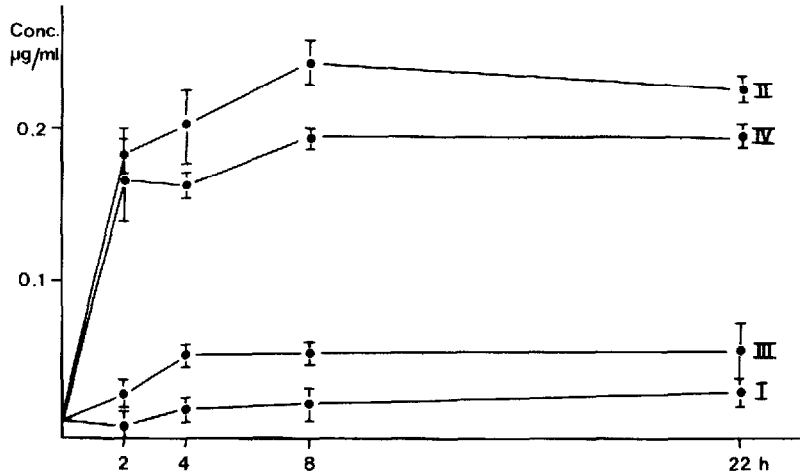


Fig. 3. Plasma benzarone concentration—incubation time curves: (I) without enzymes; (II) with β-glucuronidase/arylsulphatase, (III) with β-glucuronidase; (IV) with arylsulphatase.

phatase (Fig. 3 curves III and IV) permitted the evaluation of the relative amounts of unconjugated, glucuronidated and sulphated benzarone as 6:12:82 on a molar basis. Retention times were 5.8 and 7.4 min for the internal standard and benzarone. Identification of the benzarone HPLC peak was performed by isolating the peak fraction, methylation and GC—EI-MS (Fig. 5a) (see description of methods in Experimental). The mass spectrum was identical with authentic 4-O-methyl benzarone (Fig. 1; 4). Some characteristic peaks are: at *m/e* 280 the molecular ion ($M^{+\cdot}$); 265 ($M^{+\cdot} - 15$; loss of methyl from the ethyl side-chain in ring B); 249 ($M^{+\cdot} - 31$; loss of methoxy group in ring C); 135 and 107 originate from ring C (anisoyl and methoxyphenyl ions) and 173, 144 and 115 are fragments that contain rings A and B. These assignments have been made by comparing the mass spectrum with those of the methylated and trideuteromethylated benzarones (Fig. 1; 7—11).

The calculated recoveries for benzarone and the internal standard at a concentration of 2 µg/ml were 91.5 ± 3.5 and 93.6 ± 4.1%, respectively. Plasma

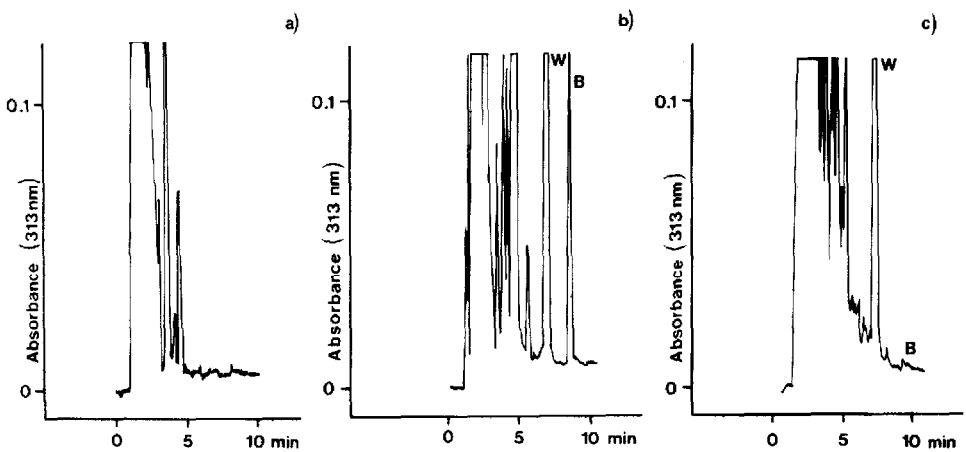


Fig. 4. HPLC of extracts from volunteer's urine. (a) Pretreatment urine; (b) urine collected 0—4 h after benzarone application and following β-glucuronidase/arylsulphatase hydrolysis; (c) as in (b) but without hydrolysis. Peaks: W = warfarin (internal standard); B = benzarone.

analyses were in the range 0.01—0.5 μg/ml with a linear calibration regression line of $y = 0.2015x + 0.0004$, (y = area benzarone/area internal standard; x = benzarone concentration in μg/ml); $r = 0.9996$; sensitivity, 0.01 μg/ml; limit of quantitation, 0.02 μg/ml.

Urine

Fig. 4a and b shows chromatograms of extracts from pretreatment urine, and urine from volunteers treated with benzarone; similarly to plasma, only a small amount of benzarone is in the unconjugated form (Fig. 4c). The relative amounts of unconjugated drug and glucuronidated and sulphated metabolites have been obtained in a way similar to that from plasma with analogous results. Benzarone peak identification after isolation by HPLC, derivatization and GC—EI-MS was performed as for plasma, and the mass spectrum was identical with that of authentic material. Extraction yields, concentration ranges and sensitivities were of the same order as those for plasma. Accuracy and precision for plasma and urine are given in Table I.

TABLE I

REPRODUCIBILITY OF PLASMA AND URINE VALUES FOR BENZARONE

Sample	Concentration added (μg/ml)	Concentration found (μg/ml)	Coefficient of variation ($n = 5$) (%)
Plasma	0.05	0.049	5 3
	0.2	0.22	2.0
	0.5	0.49	4.4
Urine	0.05	0.047	4.5
	0.2	0.18	2.8
	0.5	0.52	3.5

Validity. Plasma levels of total benzarone from eight normal volunteers were obtained after 100 mg drug intake. Fig. 5 shows the concentration—time curve obtained from a normal volunteer after a single dose: 0.73 ± 0.66% of the administered dose was eliminated in 24-h urine, showing wide inter-individual variations.

Plasma concentrations obtained by HPLC were compared with those obtained with with GC—EI-SIM-MS after methylation (see Experimental) (Fig. 3b) with good correlation ($r = 0.988$).

Interferences

No interferences in the assay for plasma and urine were observed with the substances listed in ref. 14.

DISCUSSION

In previous pharmacokinetic work with benzarone, plasma and urine [11] or urine [10] were hydrolysed with β-glucuronidase exclusively; therefore only a small portion of the total amount of benzarone could probably be determined.

A new HPLC method was developed based on our assay for the analysis of phenprocoumon [14] following hydrolysis with β-glucuronidase/arylsulphatase

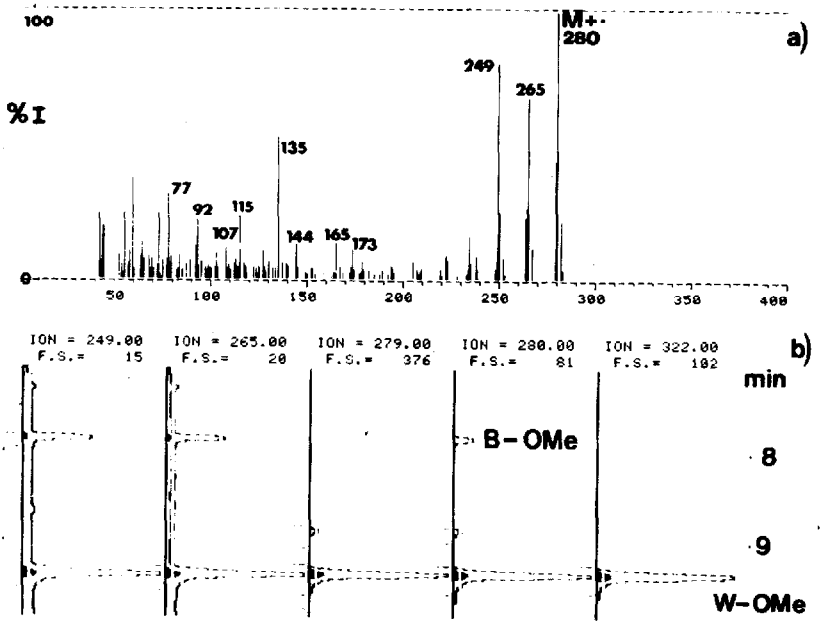


Fig. 5. GC—EI-MS spectra of benzarone plasma extracts after methylation. (a) Mass spectrum after micropreparative HPLC; (b) SIM chromatogram: B-OMe = benzarone methyl ether (Fig. 1; 4); W-OMe = warfarin methyl ether.

and extraction with 1,2-dichloroethane, after previously published methods had been evaluated [8, 9, 11]. The GC—MS technique [8], although specific and sensitive, is lengthy for routine use. The HPLC assay [9], based on methanol precipitation and isocratic elution proved to be unspecific due to interfering peaks. Lücker and Wetzelberger [11] determined benzarone after hydrolysing the samples with pure β-glucuronidase, extraction and multiple-step HPLC gradient elution using benzbromarone as internal standard; as we have shown, their method did not take into account benzarone as its sulphated conjugate.

The gradient elution programme was simplified and allowed the elimination of interfering peaks; the use of warfarin instead of benzbromarone allows the extension of our method to the quantitation of benzbromarone and metabolites in future work [12]. Filtration of samples through C_{18} cartridges [15, 16] or the use of other organic solvents [8, 11] leads to inconsistent results or interfering peaks during analysis.

Methylation of benzarone for GC—MS was preferred to trifluoracetylation [8] for its simplicity, high yields, stability of the derivatives and sharp separations by capillary GC—MS (Fig. 5b). It was used for identification of benzarone HPLC peaks and validation of plasma concentrations. MS assignments (see Results) of some of the peaks could be effected by correlation of peaks from benzarone with those from brominated and deuterated derivatives (Fig. 1; 7—11).

By using specific hydrolytic enzymatic procedures (Fig. 3) we were able to demonstrate that the drug was present in vivo mainly as sulphate and β-glucuronide (Fig. 1; 5 and 6); unconjugated benzarone was found at very low concentrations in both plasma and urine (Figs. 2c, 3 and 4).

We found that 0.73% of the ingested benzarone is eliminated conjugated in

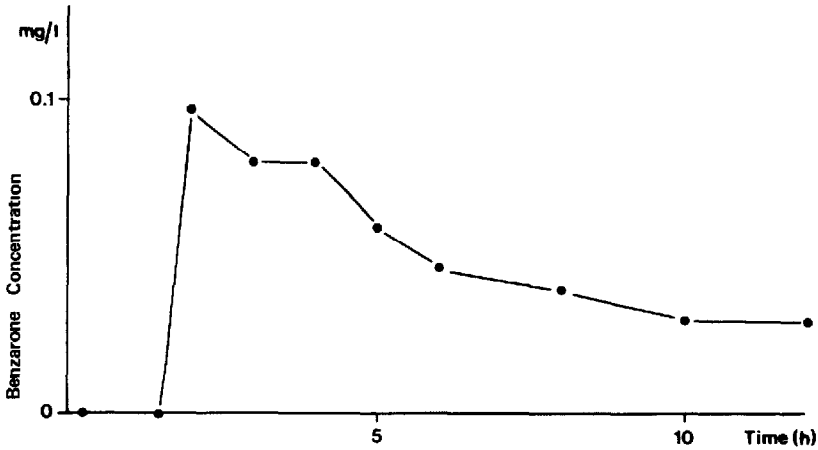


Fig. 6. Plasma benzarone concentration—time curve in a volunteer after an oral dose of 100 mg of benzarone.

urine. As it has been reported [10] that 70% of the radioactivity of a tritium-labelled drug is excreted in urine and 20% in faeces, further work should be undertaken to clarify the metabolic pathway of the drug in humans.

In conclusion, a method for the quantitative determination of total benzarone in plasma and urine after enzymatic hydrolysis has been described, which is reliable, selective and sensitive, and which may be used for clinical pharmacological investigations. We have demonstrated that unconjugated benzarone is found in only small amounts, glucuronidated and sulphated compounds representing the major part of the substance in both human blood and urine.

REFERENCES

1 R.C. Heel, R.N. Brogden, T.M. Speight and G.S. Avery, Drugs, 14 (1977) 349.
2 J. Broekhuysen, M. Pacco, R. Sion, L. Demenlenaere and M. van Hee, Eur. J. Clin. Pharmacol., 4 (1972) 125.
3 H. Ferber, H. Vergin and G. Hitzenberger, Eur. J. Clin. Pharmacol., 19 (1981) 431.
4 G. Barchewitz, in N. Klüken and R.E. Schmutzler (Editors), Ergebnisse der Angiologie, F.K. Schattauer, Stuttgart, New York, 1975, p. 119.
5 J. Goethals, in N. Klüken and R. Schmutzler (Editors), Ergebnisse der Angiologie, F.K. Schattauer, Stuttgart, New York, 1975, p. 125.
6 H. Mörl, C. Diehm, R. Zimmermann, G. Schöffel and E. Walter, Münch. Med. Wschr., 122 (1980) 277.
7 T. Yu, J. Rheumatol., 3 (1976) 305.
8 W. Stüber and H. Möller, J. Chromatogr., 224 (1981) 327.
9 H. Vergin and G. Bishop, J. Chromatogr., 183 (1980) 383.
10 J. Broekhuysen, in S. Klüken and R. Schmutzler (Editors), Ergebnisse der Angiologie, F.K. Schattauer, Stuttgart, New York, 1975, p. 97.
11 W Lücker and K. Wetzelberger, Arzneim. Forsch., 29 (I) (1979) 842.
12 I. Walter-Sack, M. Kohlmeier, J.X. de Vries, A. van de Loo and E. Weber, in preparation.
13 H.U. Bergmeyer, J. Bergmayer and M. Grassl, Methods of Enzymatic Analysis, Vol. 2. Samples, Reagents, Assessment of Results, Verlag Chemie, Weinheim, Florida, Basel, 3rd ed., 1983, pp. 157—206.
14 J.X. de Vries, J. Harenberg, E. Walter and M. Simons, J. Chromatogr., 231 (1982) 83.
15 J.X. de Vries, M. Simon, R. Zimmermann and J. Harenberg, J. Chromatogr., 338 (1985) 325.
16 J.X. de Vries, R Zimmermann and J. Harenberg, Eur. J. Clin. Pharmacol., 29 (1986) 591.