ELSEVIER

Journal of Chromatography B, 754 (2001) 209–215

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

High-performance liquid chromatography assay for simultaneous determination of dextromethorphan and its main metabolites in urine and in microsomal preparations

El-khalil Bendriss[a], Nektaria Markoglou[a], Irving W. Wainer[a,b]

[a]*Department of Oncology, Pharmacokinetics Division, McGill University, Montréal, Québec, Canada*
[b]*Department of Pharmacology, Room C-305 Medical Dental Building, Georgetown University, 3900 Reservoir Road, N.W., Washington, DC, USA*

Received 22 September 2000; received in revised form 22 November 2000; accepted 22 November 2000

Abstract

An HPLC method has been developed and validated for the determination of dextromethorphan, dextrorphan, 3-methoxymorphinan and 3-hydroxymorphinan in urine samples. Deconjugated compounds were extracted on silica cartridges using dichloromethane/hexane (95:05, v/v) as an eluent. Chromatographic separation was accomplished on a Phenyl analytical column serially connected with a Nitrile analytical column. The mobile phase consisted of a mixture of an aqueous solution, containing 1.5% acetic acid and 0.1% triethylamine, and acetonitrile (75:25, v/v). Compounds were monitored using a fluorescence detector. Calibration curves were linear over the range investigated (0.2–8.0 μM) with correlation coefficients >0.999. The method was reproducible and precise. Coefficients of variation and deviations from nominal values were both below 10%. For all the analytes, recoveries exceeded 77% and the limits of detection were 0.01 μM. The validated assay proved to be suitable for the determination of DEM metabolic indexes reported to reflect the enzymatic activity of the cytochrome P450s, CYP2D6 and CYP3A, both in vivo, when applied to urine samples from patients, and in vitro, when applied to samples from the incubation of liver microsomes with dextromethorphan.

Keywords: Dextromethorphan; Dextrorphan; 3-Methoxymorphinan; 3-Hydroxymorphinan

1. Introduction

Dextromethorphan (DEM) is a non-narcotic antitussive drug which is essentially metabolized by *N*, *O*-demethylations to form dextrorphan (DOR), 3-methoxymorphinan (3MM) and 3-hydroxymorphinan (3OM) (Fig. 1) [1,2]. *O*-Demethylations are catalyzed by the polymorphic cytochrome P-450 CYP2D6 [3–5]. *N*-Demethylations are primarily catalyzed by the cytochrome P450 CYP3A, although other CYPs may contribute [4,5]. DEM is commonly used as a probe drug for both in vitro and in vivo drug metabolism investigations in humans [3–9]. In vivo, the urinary molar ratios of DEM/DOR and DEM/3MM are used to assess the metabolic activity of CYP2D6 and CYP3A, respectively. Both enzymes are responsible for the metabolism of a large group of clinically important drugs [10].

*Corresponding author. Tel.: +1-202-687-1650; fax: +1-202-687-5015.

E-mail address: waineri@gunet.georgetown.edu (I.W. Wainer).

0378-4347/01/$ – see front matter
PII: S0378-4347(00)00609-5

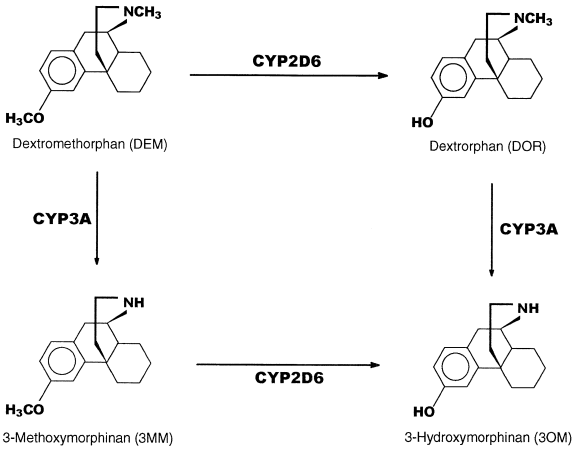

Fig. 1. Metabolic pathways of dextromethorphan to its *O*-demethylated and *N*-demethylated metabolites.

Several chromatographic methods have been reported for the determination of DEM and DOR in biological matrices. These include high-performance liquid chromatography (HPLC) [11,12], gas chromatography [13] and capillary electrophoresis [14]. For HPLC assays, numerous modes of detection have been employed including ultraviolet [11], fluorescence [11] or mass spectrometric detection [12]. Most of these reported analytical procedures were only validated for the determination of DEM and DOR. The resolution of 3MM and 3OM, which are probably systematically co-extracted under reported procedures, was rarely investigated.

The HPLC assays allowing for the simultaneous determination of DEM, DOR, 3MM and 3OM are mostly based on the methods reported by Park et al. [15], and by Chen et al. [16]. The HPLC assay with UV detection developed by Park et al. [15] consisted of urine cleaning with an extraction cartridge followed by a liquid–liquid extraction. Two extraction-analysis runs have to be conducted in order to measure DEM and its three main metabolites. The limit of quantification (LOQ) obtained by the authors ranged from 0.4 to 0.8 μ*M*, and recoveries in the range of 60–80%. Another method reported by Chen et al. [16] was validated for urine and plasma samples with recoveries of analytes ranging from 57 to 77%. This method seems to be sensitive with the limit of detection (LOD) varying from 2 to 4 n*M*. However, this method was not reproducible according to Marshall et al. [17], who failed to distinctly separate the DEM peak from the solvent front. This problem was occasionally encountered with our previously reported method [9]. The solid–liquid extraction procedure was also found relatively long for routine usage.

We report a simple, sensitive and reproducible HPLC assay for simultaneous determination of DEM, DOR, 3MM and 3GM in urine. We developed a simple and fast extraction method, using extraction silica cartridge, to increase recovery and selectivity, and to reduce time handling. We used two serially coupled analytical columns to improve separation, and optimized the analytical conditions to maintain a reasonable run time. The assay was validated for urine and applied to the analysis of urine samples from clinical studies. Samples from the in vitro incubation of the DEM with liver microsomes were also examined.

2. Experimental

2.1. Chemicals

DEM, β-glucoronidase (Type IX-A), sodium acetate, and sodium carbonate were purchased from Sigma Chemical Co., (St. Louis, MO, USA). DOR, 3MM and 3OM were obtained from Hoffman-LaRoche (Mississauga, ON, Canada). Levallorphan was provided by the USP Reference Standard (Rockville, MS, USA). One-ml ChemElut extraction silica cartridges were purchased from Varian Canada Ltd., (Mississauga, ON, Canada). HPLC-grade dichloromethane, hexane, hydrochloride, glacial acetic acid, triethylamine and acetonitrile were obtained from J.T. Baker (Phillipsburg, NJ, USA).

2.2. Apparatus

The chromatographic HPLC system included a Spectra-Physics P200 pump (Spectra-Physics, San Jose, CA, USA), a Spectra-Physics SP8875 autosampler equipped with a 100-μl loop, a Phenyl Spherisorb Reversible 5 μm column (15 cm×4.6 mm I.D.) (Regis, Chemical Co., IL, USA) serially connected with a Nitrile 5 μm column (25 cm×4.6 mm I.D.) (Regis, Chemical Co., IL, USA), and an ABI fluorescence detector (ABI Analytical, Ramsy, NJ). The detector was operated at excitation wave-

length of 200 nm and no cut-off filter was used for emission. The mobile phase was a mixture of distilled water containing 1.5% glacial acetic acid and 0.1% triethylamine, and acetonitrile (75:25, v/v) with a flow rate of 1 ml/min (the backpressure was ~2500 p.s.i.). Mobile phase was filtered through a 0.45 μm nylon membrane (Millipore Filter Corporation-Bedford, MA, USA) and sonicated prior to use for 15 min.

2.3. Sample preparation

To 250 μl of urine in a 5 ml snap-cap centrifuge tube was added 250 μl of sodium acetate buffer (0.1 *M*, pH 5), and 100 μl β-glucoronidase (Type IX-A, 1263.5 units/100 μl in sodium acetate buffer [0.1 *M*, pH 5]). The sample was gently mixed with a hand vortex mixer, the tubes were capped and incubated in a water bath at 37°C for 18 h to ensure complete hydrolysis of conjugated metabolites. After cooling, 250 μl of sodium carbonate buffer (1 *M*, pH 9.2) and 100 μl of internal standard (IS, 5 μ*M* Levallorphan in water) were added. The volume was adjusted to 1 ml with distilled water, vigorously mixed, and applied to 1-ml ChemElut extraction silica cartridge. After 5 mm, the compounds of interest were eluted into a clean glass tube with 2×4 ml of dichloromethane/hexane mixture (95:05, v/v) and the eluent was evaporated to dryness using a speed-vac. The extract was reconstituted with 250 μl of elution solvent, and 100 μl was injected into the HPLC system.

2.4. Standard solutions

Stock solutions of DEM, DOR, 3OM and 3MM were prepared in distilled water at a concentration of 1 m*M* free base. A stock solution of IS was prepared in distilled water at a concentration of 1 m*M* free base. All stock solutions were stored at 4°C. Preliminary experiments showed that stock solutions are stable for a minimum of a 6 month period under these conditions.

2.5. Method validation

For calibration curves and quality controls (QCs), samples were made by spiking drug-free urine with a known amount of stock solutions. Five concentrations in the range of 0.2–8.0 μ*M* were used to construct the calibration curve for each analyte. New standard curves were performed with each sample batch to minimize analytical variability. Calibration curves were generated by plotting the ratio of the peak area of the analyte and IS against theoretical concentrations. Correlation coefficients and the equations describing the calibration curves were determined by linear regression analysis.

Three quality control samples were prepared. The concentration was equal to 1.2, 3.2 and 6.4 μ*M* for the low quality control (LoQC), the medium quality control (MeQC) and the high quality control (HiQC), respectively. The intra- and inter-day accuracy and precision of the method were performed using five and fifteen replicates of each quality control, respectively.

The recovery of each compound was measured in triplicate by comparing peak-area of extracted quality control with the peak-area of the corresponding unextracted standards. The LOQ was set at the lowest concentration of the calibration curve. The LOD, defined as three times the signal-to-noise ratio, was determined by injecting diluted solutions of a mixture of DEM and its metabolites.

2.6. Application of the method

The method developed is being used in our laboratory for both in vivo and in vitro drug metabolism investigations. The in vivo phenotyping procedure consisted of collecting a pre-dose morning blank urine, providing the subject with 30 mg of DEM orally (Robitussin DM), and collecting a urine sample 8 h after DEM administration. On retrieval, the urine samples were stored at −20°C until analysis. For the in vitro experiments, microsomes were prepared according to the procedure described by Dayer et al. [7]. DEM incubation with microsomes was carried out as reported previously [8]. The reaction was stopped by the addition of 20 μl perchloric acid (60%). Samples were immediately vortexed for 15 s and centrifuged at 1000 *g* for 5 min, and a 100 μl aliquot of the supernatant was analyzed under the same chromatographic conditions as described for the urine samples.

3. Results and discussion

The separation achieved using the experimental conditions of the present assay for DEM, its main metabolites and the IS is presented in Fig. 2. The analysis of blank urine extracts from patients with different diseases and receiving different therapies demonstrated that no impurities co-eluted with the compounds of interest (Figs. 3 and 4). Two unidentified peaks (a & b; Figs. 3B and 4B) were detected in the urine of most of the patients having received DEM. In some probe drug metabolism phenotyping protocols, DEM is coadministered with other probe drugs such as caffeine [18,19]. The two patients included in this study received a dose of caffeine in addition to their DEM dosing. During our preliminary studies (data not shown), a mixture of caffeine and 14 of its main metabolites were analyzed under the present chromatographic conditions. The results displayed no interference of caffeine and its metabolites with the determination of 3OM, DOR, IS, 3MM or DEM. As such, the two unidentified peaks (a & b; Figs. 3B and 4B) are likely to correspond to other DEM metabolites. A previous report by Koppel et al. [20] demonstrated that the use of mass spectrometric techniques allows for the identification of more than 15 DEM metabolites in urine.

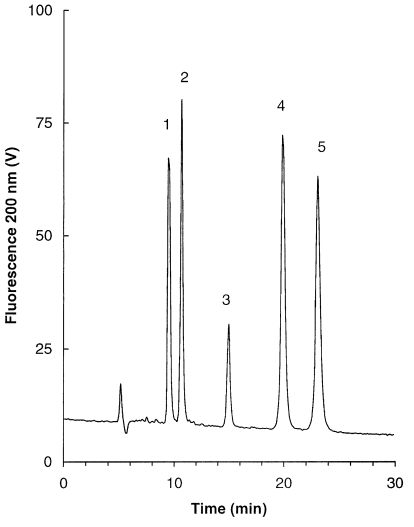

Fig. 2. Chromatogram from the analysis of a standard mixture of DEM, its *N*,*O*-demethylated metabolites, and IS. Peaks: 1=3OM (10 μ*M*), 2=DOR (10 μ*M*), 3=IS (5 μ*M*), 4=3MM (10 μ*M*), 5=DEM (10 μ*M*).

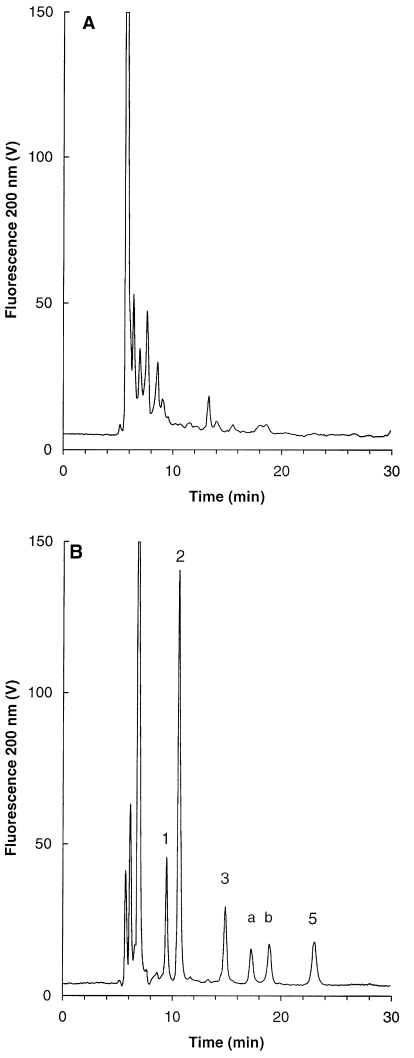

Fig. 3. Chromatograms from the analysis of urine samples from an HIV patient: (A) blank urine, (B) 8-h urine sample after the oral administration of 30 mg of DEM. Peaks: 1=3OM, 2=DOR, 3=IS, 5=DEM, a and b=unidentified peaks.

Chromatograms obtained from the analysis of the in vitro incubation mixture are free from impurities that may coelute with the analytes (Fig. 5). Therefore, an extraction is not necessary for the analysis of samples from the in vitro incubations of DEM. The two unidentified peaks (a & b; Figs. 3B and 4B) detected in the urine samples were not present in the

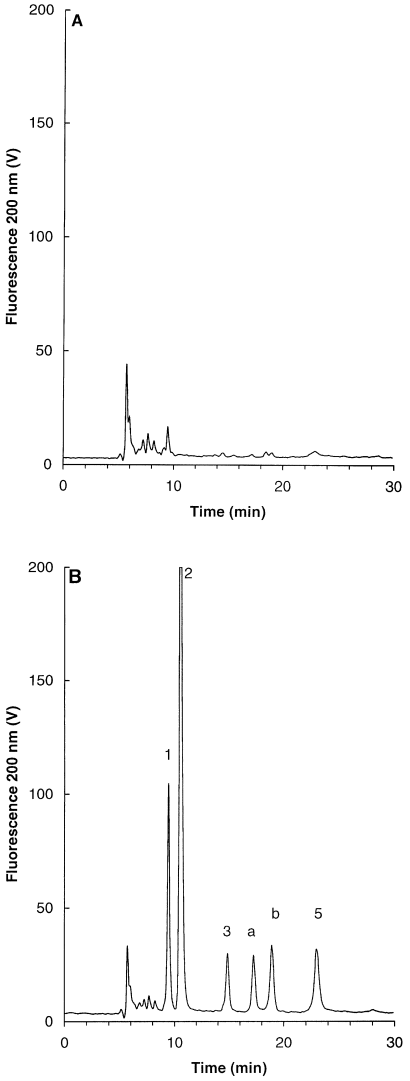

Fig. 4. Chromatograms from the analysis of urine samples from a malaria patient: (A) blank urine, (B) 8-h urine sample after the oral administration of 30 mg of DEM. Peaks: 1=3OM, 2=DOR, 3=IS, 5=DEM, a and b=unidentified peaks.

in vitro incubations of DEM (Fig. 5C). As described by Koppel et al. [20], the metabolic pathways of DEM are *O*,*N*-demethylation, *O*,*N*-acetylation, hydroxylation of the phenyl ring and the saturated ring system with subsequent further oxidation. The DEM in vitro assay has been validated only for the *O*,*N*-demethylation pathways. It can be theorized, that if the two unidentified peaks (a & b; Figs. 3B and 4B) are indeed DEM metabolites, the present conditions of the DEM in vitro assay do not allow for their production. Another explanation may be the lack of cytosolic enzymes responsible for the formation of these DEM metabolites. The metabolic reactions which are dependant upon the cytosolic enzymes are absent in the microsome metabolism model.

For each sample batch, the calibration curves for DEM, DOR, 3MM and 3OM were linear within the range investigated (0.2–8.0 μM). The standard curves were designed to address the expected range of concentrations commonly encountered in 8h-urine samples after an administration of a standard dose (20–30 mg) of DEM [17,21]. Correlation coefficients (r^2) were >0.999 for all quantified compounds. For each QC, the concentration was recalculated from the equation of the linear regression curve.

The results of intra-day ($n=5$) and inter-day ($n=15$) validation are summarized in Table 1. The method is reproducible with coefficient of variation (C.V.) values between 1.02 and 8.20% for the intra-day validation, and between 3.07 and 9.58% for the inter-day validation. The present method yields standard accuracy values from 93.62 to 106.90%, and from 92.79 to 100.80% during intra-day and inter-day validation, respectively, at all the concentrations investigated.

The respective recoveries for DEM, DOR, 3MM and 3OM obtained with the present extraction procedure are summarized in Table 1. The different QC concentrations gave rise to percent recoveries $>77\%$. The LOD of each compound was 0.01 μM. The LOQ of the four analytes was determined to be 0.2 μM. Recoveries reported by previous methods were mostly comprised between 77 and 46%, and LOQs achieved using UV or fluorescence detection were ranged from 0.4 to 1 μM [11,15,17,22].

Debrisoquine [23,24] and sparteine [25,26] have been extensively used for CYP2D6 phenotyping. The use of these probe drugs became restricted due to their potential side-effects. DEM, which is sold over the counter, has been proposed as an alternative for CYP2D6 phenotyping studies both in vitro and in vivo [3–9]. DEM has been speculated to provide the opportunity to monitor CYP3A activity [4,5,9]. When incubated with human liver microsomes, DEM was found to be more specific for CYP2D6 than debrisoquine or sparteine, and therefore, conducting in vitro metabolic studies with DEM requires a

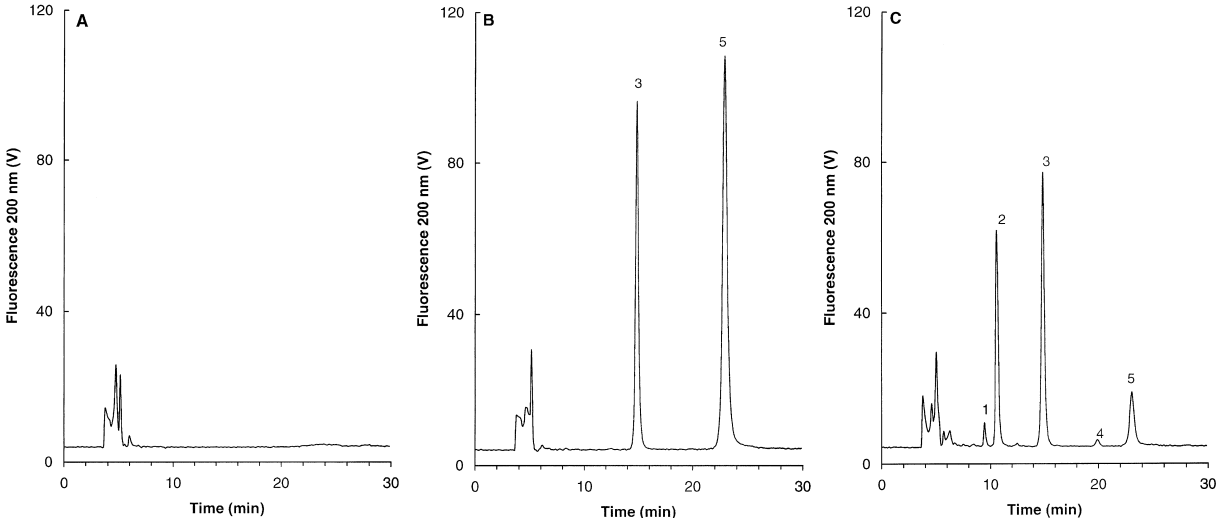

Fig. 5. Chromatograms from the analysis of samples from the in vitro incubation of DEM (20 μ*M*) with liver microsomes (50 μg) for 30 min at 37°C: (A) incubation in the absence of DEM, (B) incubation in the absence of microsomes, (C) formation of DEM metabolites in the microsomal preparation. Peaks: 1=3OM, 2=DOR, 3=IS, 4=3MM, 5=DEM.

Table 1
Results of intra- and inter-day validation in spiked human urine[a]

	LoQC				MeQC				HiQC			
	3OM	DOR	3MM	DEM	3OM	DOR	3MM	DEM	3OM	DOR	3MM	DEM
Nominal concentration (μ*M*)	1.20				3.20				6.40			
Intra-day validation												
n	5				5				5			
Measured mean concentration (μ*M*)	1.25	1.12	1.16	1.12	3.26	3.42	3.07	3.17	6.45	6.51	6.40	6.40
SD (μ*M*)	0.10	0.05	0.03	0.05	0.10	0.14	0.08	0.03	0.08	0.10	0.14	0.14
C.V. (%)	8.20	4.60	2.69	4.65	3.11	4.11	2.45	1.02	1.18	1.56	2.23	2.19
Accuracy (%)	104.09	93.62	96.82	93.71	101.99	106.90	96.04	98.95	100.72	101.75.	100.04	99.94
Inter-day validation												
n	15				15				15			
Measured mean concentration (μ*M*)	1.21	1.12	1.17	1.18	3.06	3.15	2.97	3.02	6.08	6.36	6.11	6.10
SD (μ*M*)	0.07	0.03	0.05	0.07	0.23	0.30	0.18	0.20	0.32	0.34	0.42	0.35
C.V. (%)	6.14	3.07	4.05	5.57	7.35	9.58	6.19	6.55	5.21	5.33	6.87	5.67
Accuracy (%)	100.80	93.13	97.54	98.38	95.75	98.50	92.79	94.53	95.02	99.45	95.44	95.35
Recovery (%)	88	98	82	89	84	84	80	89	78	89	78	83

[a] Limit of detection is 0.01 μ*M* for the four analytes.

limited amount of liver microsomes. This is an important aspect that might contribute to reducing the need of healthy human liver samples, which become less available with the important development of liver transplantation.

In clinical drug metabolism investigations, the DEM probe drug is usually added to the scheduled treatment of the patient. In order to minimize the risk of interference between analytes and other drugs or endogenous compounds, a simple extraction procedure is always recommended. In other respects, some individuals might have a very low amount of DEM or 3MM. A simple and sensitive assay will prove useful, for example, in phenotyping individuals with extremely high CYP2D6 activity who may have low urinary DEM concentrations.

The present assay is based on a simple extraction and analysis procedure using a conventional HPLC-fluorescence detector system. The good specificity and sensitivity obtained are mainly attributable to the introduction of the extraction step, using silica extraction cartridge, which allow for the selective and the high recovery of DEM and its metabolites. Another parameter that resulted in increased specificity is the use of β-glucuronidase from *Escherichia Coli* instead of β-glucuronidase from *Helix Pomatia*. When applied to the analysis of urine samples from patients (Figs. 3 and 4) or to the analysis of samples from the incubation of DEM with liver microsomes (Fig. 5), the results show that the developed assay is sensitive and convenient for routine determination of metabolic indexes reported to reflect in vitro or in vivo CYP2D6 and CYP3A enzyme activities.

Acknowledgements

This research was supported by a Strategic Grant in Nutrition and Cancer number 3263 to Irving W. Wainer from the Cancer Research Society Inc., Canada. We gratefully acknowledge Paul Drogaris for his assistance in the in vitro incubations.

References

[1] J.W. Barnhart, Toxicol. Appl. Pharmacol. 55 (1980) 43.
[2] G. Pfaff, P. Briegel, I. Laniprecht, Int. J. Pharm. 14 (1983) 173.
[3] B. Schmid, J. Bircher, R. Preisig, A. Küpfer, Clin. Pharmacol. Ther. 38 (1985) 618.
[4] L.L. Von Moltke, D.J. Greenblatt, J.M. Grassi, B.W. Granda, K. Venkatakrishnan, I. Schmider, J.S. Harmatz, R.I. Shader, J. Pharm. Pharmacol. 50 (1998) 997.
[5] J. Schmider, D.J. Greenblatt, S.M. Fogelman, L.L. Von Moltke, R.I. Shader, Biopharm. Drug Dispos. 18 (1997) 227.
[6] A. Küpfer, B. Schmid, G. Pfaff, Xenobiotica 16 (1986) 421.
[7] P. Dayer, T. Leemann, R. Striberni, Clin. Pharmacol. Ther. 45 (1989) 34.
[8] A. Bendriss, Y. Bechtel, G. Paintaud, E.K. Bendriss, C. Joanne, S. Bresson-Hadni, J. Magnette, M.C. Becker, M. Gillet, G. Mantion, J.P. Miguet, P.R. Bechtel, Ther. Drug Monit. 17 (1995) 113.
[9] J. Ducharme, S. Abdullah, I.W. Wainer, J. Chromatogr. B Biomed. Appl. 678 (1996) 113.
[10] D.D. Breimer, in: Office for Official Publications of the European Communities editors, European Conference on Specificity and Variability in Drug Metabolism, Brussels–Luxembourg: ECSC-EC-EAEC: 1995, p. 1.
[11] Y.W. Francis Lam, S.Y. Rodriguez, Ther. Drug Monit. 15 (1993) 300.
[12] T.H. Eichhold, L.J. Greenfield, S.H. Hoke II, K.R. Wehmeyer, J. Mass Spectrom. 32 (1997) 1205.
[13] P. Baumann, M. Jonzier-Perey, Clin. Chim. Acta 171 (1988) 211.
[14] J. Caslavska, E. Hufschmid, R. Theurillat, C. Desiderio, H. Wolfisberg, W. Thormann, J. Chromatogr. B Biomed. Appl. 656 (1994) 219.
[15] Y.H. Park, M.P. Kullberg, O.N. Hinsvark, J. Pharm. Sci. 73 (1984) 24.
[16] Z.R. Chen, A.A. Somogyi, F. Bochner, Ther. Drug Monit. 12 (1990) 97.
[17] P.S. Marshall, R.J. Straka, K. Johnson, Ther. Drug Monit. 14 (1992) 402.
[18] W.M. O'Neil, B.M. Gilfix, N. Markoglou, A. Digirolamo, C.M. Tsoukas, I.W. Wainer, Eur. J. Clin. Pharmacol. 56 (2000) 321.
[19] W.M. O'Neil, B.M. Gilfix, A. Digirolamo, C.M. Tsoukas, I.W. Wainer, Clin. Pharmacol Ther. 62 (1997) 261.
[20] C. Koppel, J. Tenczer, K. Ibe, Arzneimittelforschung 37 (1987) 1304.
[21] E. Jacqz-Aigrain, Y. Menard, M. Popon, H. Mathieu, J. Chromatogr. 495 (1989) 361.
[22] D.R. Jones, J.C. Gorski, M.A. Hamman, S.D. Hall, J. Chromatogr. B Biomed. Appl. 678 (1996) 105.
[23] A. Mahgoub, J.R. Idle, L.G. Dring, R. Lancaster, R.L. Smith, Lancet 2 (1977) 584.
[24] P. Evans, A. Mahgoub, T.P. Sloan, J.R. Idle, R.L. Smith, J. Med. Genet. 17 (1980) 102.
[25] M. Eichelbaum, N. Spannbrucker, B. Steincke, H.J. Dengler, Eur. J. Clin. Pharmacol. 16 (1979) 183.
[26] T. Inaba, S.V. Otton, W. Kalow, Clin. Pharmacol. Ther. 27 (1980) 547.