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Journal of Chromatography B, 732 (1999) 185–192

JOURNAL OF CHROMATOGRAPHY B

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Direct determination of tramadol glucuronides in human urine by high-performance liquid chromatography with fluorescence detection

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Received 20 January 1999; received in revised form 17 May 1999; accepted 15 June 1999

Abstract

A sensitive and selective reversed-phase high-performance liquid chromatography method has been developed for the direct determination of three glucuronides of the centrally acting analgesic tramadol (**1**). Separation of these glucuronides into their diastereomers was achieved by HPLC using ion pair chromatography with nonanesulfonic acid sodium salt and LiChrospher 100 RP 18 as stationary phase. Quantification of *O*-demethyltramadol glucuronide and *N*,*O*-didemethyltramadol glucuronide in human urine was performed by fluorescence detection. The urine samples were purified by a two-step solid-phase extraction. The glucuronides were found to be highly enriched in the 1*S*,2*S*-diastereomers. The results of a study with three healthy volunteers are presented.

Keywords: Tramadol glucuronide

1. Introduction

Tramadol (Fig. 1), 2(e)-dimethylaminomethyl-1(e)-(3-methoxyphenyl)-cyclohexan-1-(a)-ol, a chiral cyclohexanol derivative, is an opioid-type analgesic with additional non-opioid properties [1]. The racemate is successfully used for the treatment of severe postoperative or cancer pain. The (+)-1*R*,2*R*-enantiomer of tramadol exhibited a tenfold higher analgesic activity than the (−)-1*S*,2*S*-enantiomer [2]. The metabolism of tramadol is summarised in Fig. 2. The active metabolite *O*-demethyl tramadol (**2**) has a higher analgesic potency than tramadol itself [3]. Humans excrete up to 30% of the applied dose of tramadol as conjugated phenolic metabolites in urine [4]. The phase I metabolism was found to be stereoselective but interindividual differences of the enantiomeric ratio of **1** as well as its *O*-demethylmetabolite **2** were observed in urine [5]. In vitro *O*-demethylation of **1** was highly stereoselective. The V_{max} of *O*-demethylation of (−)-1*S*,2*S*-**1** was twofold higher compared to the (+)-1*R*,2*R*-enantiomer [6].

Until now no investigation regarding the stereoselectivity of the glucuronidation of the phenolic tramadol metabolites has been published. In this communication we describe the direct simultaneous resolution of the diastereomers of *O*-demethyltramadol glucuronide (**5**), *N*,*O*-didemethyltramadol glucuronide (**6**) and *N*,*N*,*O*-tridemethyl-

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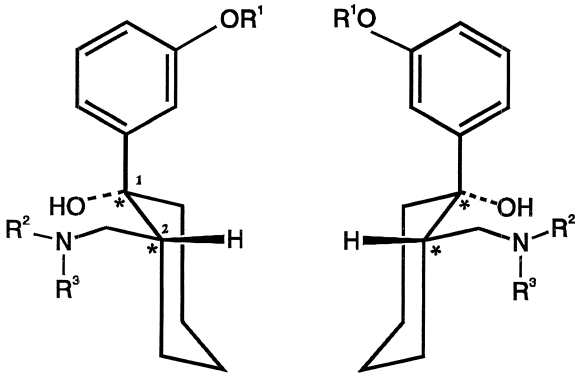
0378-4347/99/$ – see front matter
PII: S0378-4347(99)00281-9



	R^1	R^2	R^3
1	-CH_3	-CH_3	-CH_3
2	-H	-CH_3	-CH_3
3	-H	-H	-CH_3
4	-H	-H	-H
5	-GlucAc	-CH_3	-CH_3
6	-GlucAc	-H	-CH_3
7	-GlucAc	-H	-H

Fig. 1. Structures of the enantiomers of tramadol (**1**), *O*-demethyltramadol (**2**), *N*,*O*-didemethyltramadol (**3**), *N*,*N*,*O*-tridemethyltramadol (**4**) and the diastereomers of *O*-demethyltramadol glucuronide (**5**), *N*,*O*-didemethyltramadol glucuronide (**6**) and *N*,*N*,*O*-tridemethyltramadol glucuronide (**7**), GlucAc = Glucuronic acid.

tramadol glucuronide (**7**). The diastereomers of the glucuronides **5** and **6** were quantified in the urine of three volunteers upon oral administration of racemic tramadol.

2. Experimental

2.1. Materials

Tramadol hydrochloride (**1**) and its metabolite *N*,*N*,*O*-tridemethyltramadol (**4**) were supplied by Grünenthal (Stolberg, Germany). *O*-Demethyltramadol hydrochloride (**2**) and *N*,*O*-didemethyltramadol hydrochloride (**3**) had been synthesized previously by Elsing and Blaschke [5], *O*-demethyltramadol glucuronide (**5**) by Dr. J.B. Lenfers (Wuppertal, Germany) from racemic **2**.

Reagent grade methanol was purchased from Merck (Darmstadt, Germany), nonanesulfonic acid sodium salt and 3,5-dimethoxybenzaldehyde which was used as internal standard were purchased from Aldrich (Steinheim, Germany). C_{18} cartridges (Bakerbond, 100 mg sorbent, 7020-01) were generously supplied by J.T. Baker (Gross-Gerau, Germany). Cation-exchange cartridges (Chromabond SA, 500 mg sorbent, 730077) were purchased from Macherey-Nagel (Düren, Germany). All other chemicals used in the experiments were of analytical and reagent grade. Buffer solutions were prepared with deionized, doubly distilled water filtered through a 0.22 μm filter.

2.2. Preparative separation of the O-demethyltramadol enantiomers

A mixture of racemic *O*-demethyltramadol base (1.6 g, 6.3 mmol) and either *L*-(+)- or *D*-(−)-mandelic acid (1.0 g, 6.3 mmol) in anhydrous ethanol was allowed to crystallise for two days at room temperature. Two recrystallisation steps from ethanol afforded a complete separation of the diastereomeric salts. After decomposition of the salts with ammonia and extraction with ethyl acetate, both enantiomers of *O*-demethyltramadol were obtained in yields of ca. 35% and characterized by ^{1}H-NMR, ^{13}C-NMR, mass spectrometry and melting points (139–142°C). The specific rotations were $[\alpha]_D^{20} = +15.5$ and -15.5 (ethanol, $c = 0.85$). The enantiomeric purity of both enantiomers of **2** was ≥99% as assayed by HPLC analysis on a Chiralcel OD column [5,7].

2.3. Instrumentation and experimental conditions

2.3.1. HPLC

The chromatographic system consisted of a Merck Hitachi L-6000 pump (Merck), a Rheodyne sample injector (Model 7125, Rheodyne) equipped with a 50 μl loop, and a variable-wavelength fluorescence detector (Model F-1050, Merck Hitachi). The excita-

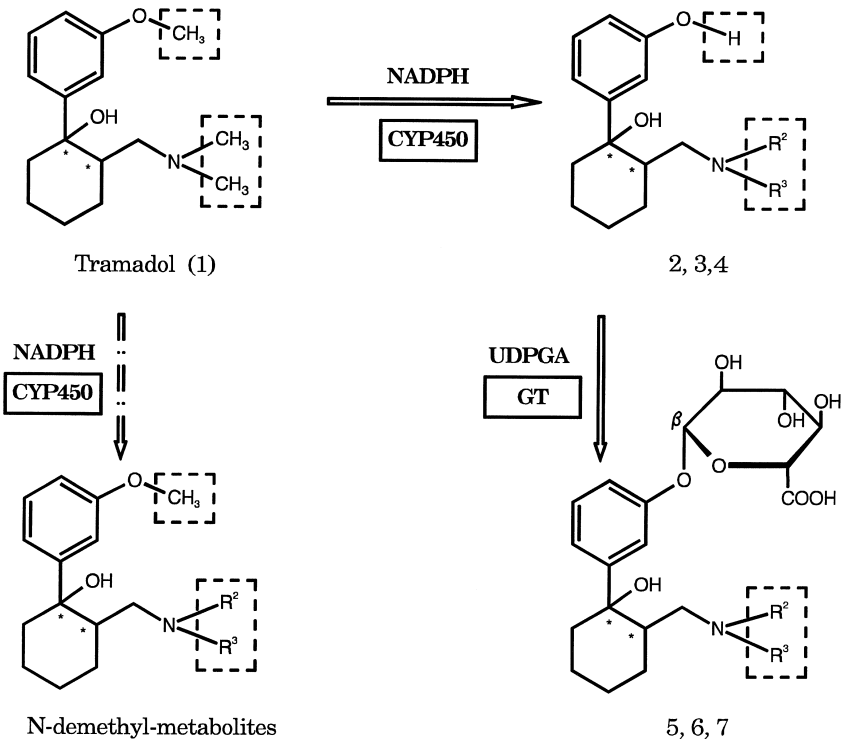


Fig. 2. Metabolism of tramadol.

tion wavelength was 270 nm, the emission wavelength was 300 nm. Data recording and processing were performed using a Merck Hitachi chromato-integrator (Model D-2000).

The separation of the glucuronides was performed using a LiChrospher 100 RP 18 (Merck, 5 μm particle size, 250 mm×4.0 mm I.D.) column equipped with a LiChrospher 100 RP 8 guard column (10 μm particle size, 4.0 mm×4.0 mm I.D.). The mobile phase consisted of 0.05 M phosphate buffer containing 0.02 *M* nonanesulfonic acid sodium salt, adjusted to pH 4.5, and 16.7% methanol at a flow-rate of 0.8 ml/min.

2.3.2. Capillary electrophoresis

Capillary electrophoresis experiments were performed using a Beckman P/ACE 2100 system (Beckman, Munich, Germany) with an uncoated fused-silica capillary. Separations were carried out with 50 m*M* phosphate buffer of pH 3.0. Samples were introduced into the capillary by pressure injection (5 s) and separated at 18.8 kV. The temperature was maintained at 20°C. Detection was carried out at a wavelength of 214 nm.

2.4. Sample preparation

The samples were purified by solid-phase extraction on a Baker spe-12G system in two steps.

C_{18} cartridges were pretreated with 2.0 ml of methanol, 1.0 ml of water and 1.0 ml of 1.0 *M* ammonium sulphate adjusted to pH 9.8 with ammonia. 0.5 ml of urine was mixed with 0.5 ml of 1.0 *M* ammonium sulphate adjusted to pH 9.8 with ammonia and applied to the C_{18} cartridge. After washing with 1 ml of 1 *M* ammonium sulphate and 1 ml of water the glucuronides were eluted with 2 ml of a mixture of 0.1 *M* phosphoric acid–methanol (7:3, v/v).

In the second purification step a cation-exchange cartridge was pretreated with 3 ml of methanol, 2 ml of water, and 1 ml of a mixture of 0.1 *M* phosphoric acid–methanol (7:3). The eluate (2 ml) from the C_{18} cartridge was passed through the cation-exchange cartridge followed by 1 ml of a mixture of 0.1 *M*

phosphoric acid–methanol (7:3), 1 ml of 0.1 *M* acetic acid, and 2 ml of methanol. The glucuronides were eluted with 2 ml of 3% ammonia in methanol.

The eluate was evaporated to dryness under a gentle stream of nitrogen. Fifty μl of the internal standard solution were added. After evaporation of the solvent the residue was dissolved in 1.0 ml of the mobile phase. Fifty μl were injected into the HPLC system.

2.5. In vivo study

Three healthy volunteers received 100 mg of tramadol hydrochloride (Tramal) p.o. Urine collection was carried out in ten intervals until 30 h after dosage. The samples were stored at −20°C until analysis. Volume and pH were determined immediately, and the samples were stored at −20°C until analysis. The glucuronides proved to be stable, and no change in the sample concentration during storage was observed.

2.6. In vitro glucuronidation by rat liver microsomes

Microsomes of liver homogenates of phenobarbitone induced Sprague Dawley rats were used for in vitro glucuronidation of the phenolic phase I metabolites **2**, **3** and **4**. Microsomal protein was determined by the method of Bradford with bovine serum albumin as standard [8].

Microsomal incubations (1-ml volume) contained UDPGA (2.5 μmol/ml), $MgCl_2$ (2.5 m*M*), Tris–HCl (25 m*M*), microsomal protein (1.7 mg/ml) and the substrates 1*R*,2*R*-**2** and 1*S*,2*S*-**2** (each 0.25 μmol/ml).

3. Results and discussion

3.1. Determination of the diastereomeric ratio of O-demethyltramadol glucuronide standard

The diastereomeric ratio of **5** was determined by the HPLC assay described above. The result was confirmed by capillary electrophoresis. A representative electropherogram is shown in Fig. 3. A ratio of

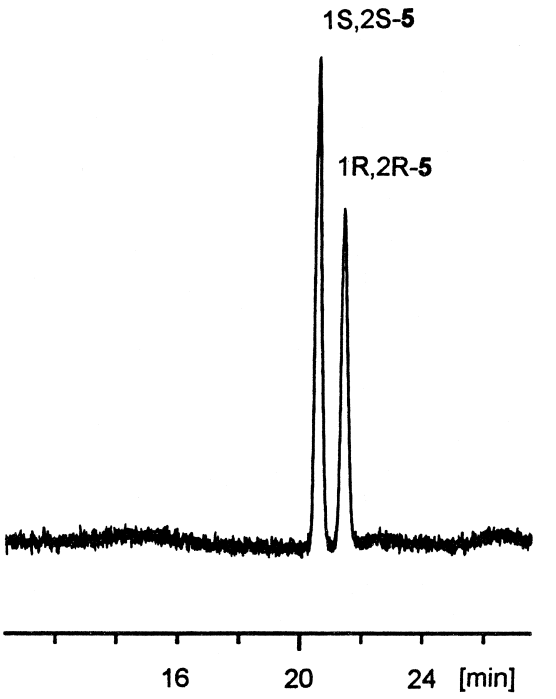


Fig. 3. Electropherogram of the diastereomeric separation of **5**. Conditions: fused-silica capillary 40/47 cm×50 μm I.D., 50 m*M* phosphate buffer pH 3.0, E=400 V/cm, 55 μA, injection by pressure, UV-detection at 214 nm.

45% of the 1*R*,2*R*-**5** to 55% of the 1*S*,2*S*-diastereomer was found. This deviation from the expected ratio of 50:50 of the diastereomeric mixture resulted from the purification step of the synthetic raw material.

By incubation of the 1*R*,2*R*- and 1*S*,2*S*-enantiomers of **2** with UDP glucuronic acid (UDPGA) the elution order of the diastereomers of the *O*-demethyltramadol glucuronide (**5**) was determined. The elution orders of the diastereomers of **6** and **7** were elucidated by incubation in two steps. In a first step either the 1*R*,2*R*- or 1*S*,2*S*-enantiomer of **2** were incubated with 2.5 μmol nicotinamide adenine dinucleotide phosphate (NADPH) in the presence of liver homogenates. To the corresponding enantiomers of **3** respectively **4** were formed. In a second step the enantiomers of **3** respectively **4** were incubated in situ with UDPGA to the corresponding diastereomers of **6** respectively **7**.

3.2. Quantitative determination of the diastereomers of the O-demethyl- and N,O-didemethyltramadol glucuronide

Precision and accuracy of the assay were determined in blank urine samples spiked with known amounts of the diastereomers of **5** in the range of 0.035–4.32 μg/ml (1*R*,2*R*-**5**) and 0.042–5.24 μg/ml (1*S*,2*S*-**5**), respectively. The results are summarised in Table 1. Each concentration was analyzed four times. A linear correlation was found:

1*R*,2*R*-**5** $y = 0.76111x - 0.01490, \quad r = 0.9996$

1*S*,2*S*-**5** $y = 0.77376x - 0.02259, \quad r = 0.9996$

Recoveries were evaluated by comparing the peak areas from urine samples spiked with different amounts of the diastereomers of **5** and those of standard solutions (Table 2). The recovery (±S.D.) of 1*S*,2*S*-**5** was 90.1±4.5%, the recovery of 1*R*,2*R*-**5** was 89.6±5.7%. Limits of detection defined as a signal-to-noise ratio of 3:1 were determined at 8.5 ng/ml for 1*R*,2*R*-**5** and 10.5 ng/ml for 1*S*,2*S*-**5**.

Because the reference substance **6** was not available quantification of its diastereomers was based on the calibration data of the diastereomers of **5**. However the fluorescence properties of the corresponding non-conjugated metabolites **2** and **3** were

Table 1
Reproducibility (intra-day) and accuracy after extraction of *O*-demethyltramadol glucuronide (**5**) from blank urine ($n=4$)[a]

	Concentration (μg/ml)	Average peak area **5**/**I.S.**	S.D.
1*R*,2*R*	0.035	0.0250	0.0012
	0.086	0.0642	0.0027
	0.539	0.3931	0.0057
	1.081	0.8070	0.0214
	2.158	1.5796	0.0684
	4.317	3.2949	0.0399
1*S*,2*S*	0.042	0.0312	0.0009
	0.105	0.0763	0.0030
	0.654	0.4749	0.0064
	1.312	0.9878	0.0279
	2.620	1.9500	0.0903
	5.240	4.0610	0.0420

[a] $n=4$; column: LiChrospher 100 RP 18, 250×4 mm I.D. (5 μm). Fluorimetric detection, $\lambda_{ex}=270$ nm, $\lambda_{em}=300$ nm.

Table 2
Recovery of the diastereomers of **5** after extraction from blank urine[a]

	Concentration (μg/ml)	Average recovery (%)
1*R*,2*R*	0.035	91.9
	0.086	93.2
	0.539	89.7
	1.081	94.9
	2.158	88.3
	4.317	82.3
1*S*,2*S*	0.042	97.5
	0.105	89.8
	0.654	87.6
	1.312	93.7
	2.620	87.8
	5.240	81.0

[a] $n=4$; column: LiChrospher 100 RP 18, 250×4 mm I.D. (5μm). Fluorimetric detection, $\lambda_{ex}=270$ nm, $\lambda_{em}=300$ nm.

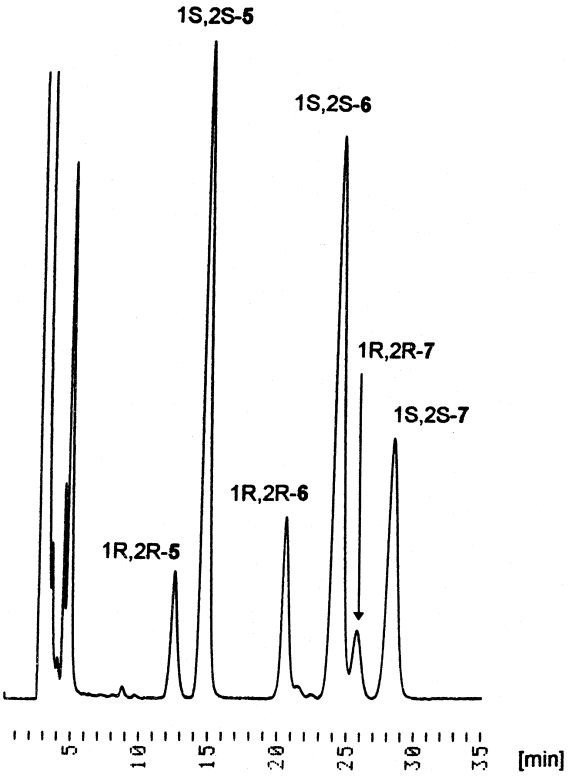


Fig. 4. Chromatogram of the diastereomeric glucuronides **5**, **6** and **7** achieved by in vitro glucuronidation of racemic **2**, **3** and **4**. Conditions: LiChrospher 100 RP 18 column, 0.05 *M* phosphate buffer pH 4.5, 0.02 *M* nonanesulfonic acid sodium salt–methanol (83.3:16.7); flow-rate 0.8 ml/min; fluorimetric detection.

comparable. Therefore it is assumed that the glucuronides **5** and **6** should also have similar fluorescence properties allowing the quantitation of **6** based on the data of **5**. The chromatogram of the glucuronides is shown in Fig. 4.

Fig. 5 shows typical chromatograms of blank urine (**a**) and an urine sample of the 10–12 h collecting interval (volunteer 1) (**b**) after oral administration of 100 mg racemic tramadol hydrochloride. The cumulative excretion of the glucuronides **5** and **6** for volunteer 3 is shown in Fig. 6. During a 30 h interval 10.6–20.2% of the applied dose were eliminated as the glucuronides **5** and **6**. Renal elimination of the glucuronides **5** and **6** was highly diastereoselective in the case of all three volunteers as summarised in Tables 3 and 4. The 1*S*,2*S*-diastereomer of both glucuronides was preferentially excreted. As shown in Table 3 only 11.9% (volunteer 1), 21.6% (volunteer 2) and 15.5% (volunteer 3) were excreted as the 1*R*,2*R*-diastereomer of the glucuronide **5**. The diastereomeric ratio (1*R*,2*R*-**5**/1*S*,2*S*-**5**) was 0.1–0.2 (volunteer 1), 0.3–0.4 (volunteer 2) and 0.2–0.3 (volunteer 3). In case of glucuronide **6**, 17.4% (volunteer 1), 16.2% (volunteer 2) and 13.6% (volunteer 3) were eliminated as the 1*R*,2*R*-diastereomer (Table 4). The diastereomeric ratio (1*R*,2*R*-**6**/1*S*,2*S*-**6**) was 0.1–0.3 (volunteer 1), 0.1–0.2 (volunteer 2) and 0.1–0.2 (volunteer 3). The diastereomers of glucuronide **7** were not found in quantifiable amounts in the urine samples of the volunteers.

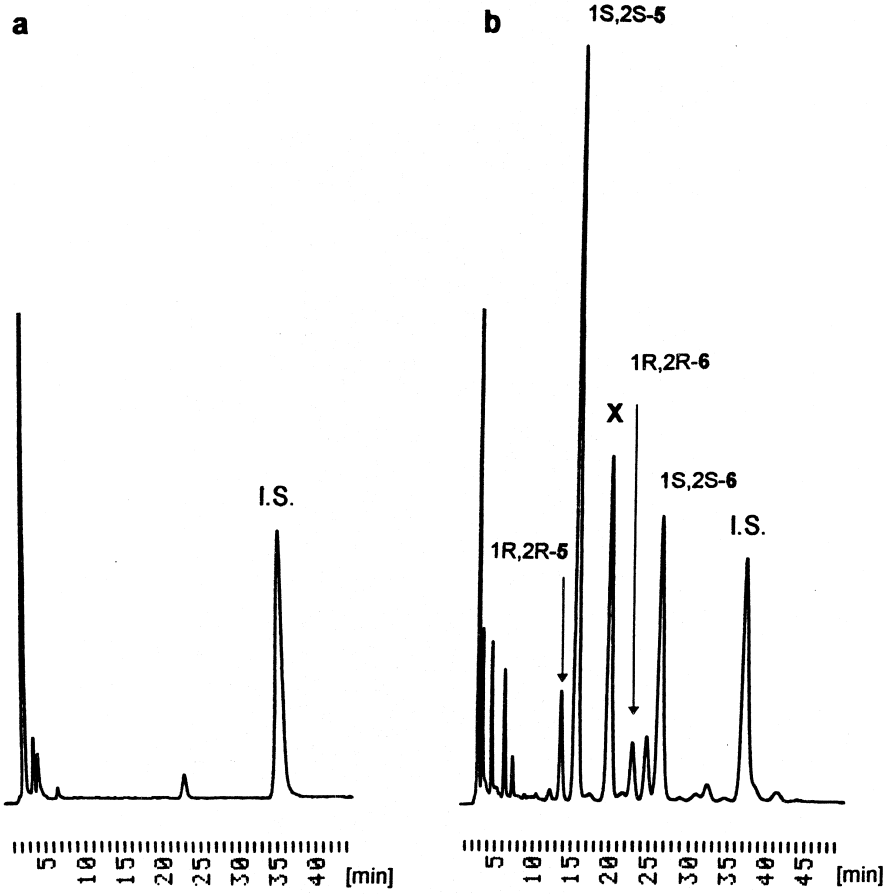


Fig. 5. Chromatograms of a blank urine spiked with the internal standard (I.S.) 3.5-dimethoxybenzaldehyde (a) and an urine sample of the collection interval 10–12 h after oral administration of 100 mg racemic tramadol hydrochloride (b). X marks an unidentified metabolite which is hydrolysed by β-glucuronidase. Chromatographic conditions are the same as in Fig. 4.

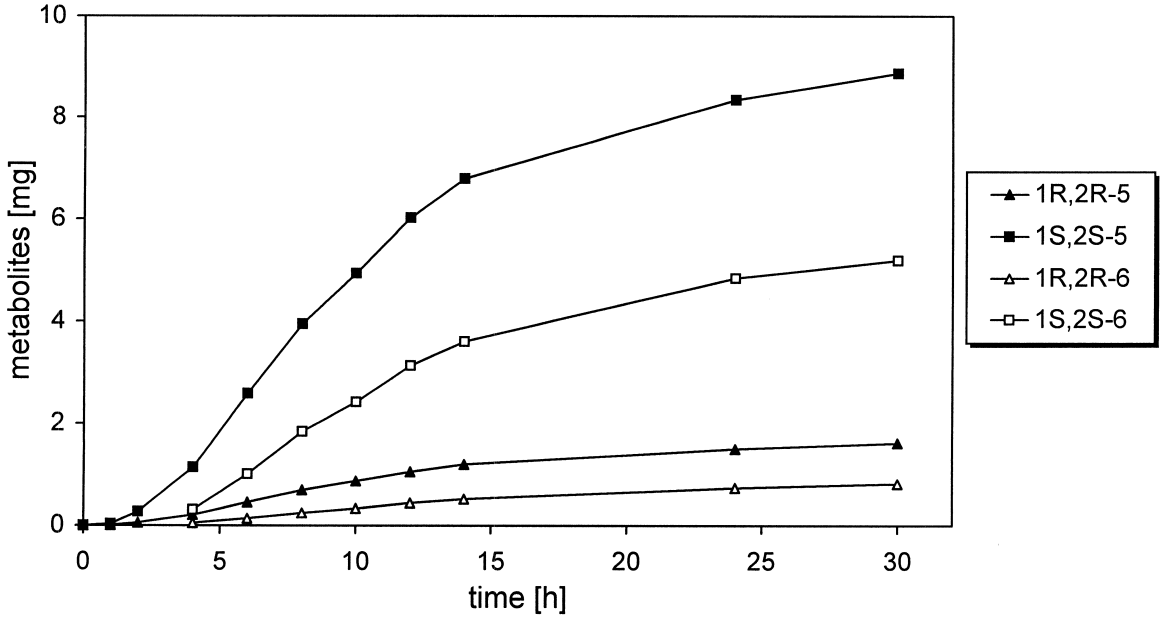


Fig. 6. Cumulative excretion of the diastereomers of *O*-demethyltramadol glucuronide (**5**) and *N*,*O*-didemethyltramadol glucuronide (**6**) after oral administration of 100 mg racemic tramadol hydrochloride to volunteer 3.

Volunteer 2 excreted more than twice the amount of both distereomers of glucuronide **5** compared to volunteers 1 and 3. This difference in the amount of glucuronide **5** and **6** in the urine samples of volunteer 2 might be due to a higher activity of glucuronosyl transferases and/or a higher formation of the phenolic metabolites **2** and **3** by CYP450 monooxygenases. The cumulative excretion of the diastereomers of **5** and **6** after oral administration of 100 mg racemic tramadol hydrochloride to volunteer 3 is shown in Fig. 6.

4. Conclusion

The diastereoselective determination of tramadol glucuronides in human urine was performed using HPLC. This assay is sensitive, reproducible and

Table 3
Cumulative excretion (μg) and diastereomeric ratio of *O*-demethyltramadol glucuronide (**5**) in urine of three volunteers after oral administration of 100 mg tramadol hydrochloride

Collection interval (h)	Volunteer 1		Volunteer 2		Volunteer 3	
	1*R*,2*R* (μg)	1*S*,2*S* (μg)	1*R*,2*R* (μg)	1*S*,2*S* (μg)	1*R*,2*R* (μg)	1*S*,2*S* (μg)
0–1	7.9	34.9	10.6	23.2	11.4	36.7
1–2	70.3	639.7	94.4	303.6	61.3	268.7
2–4	199.8	1903.1	344.9	1243.3	213.2	1142.6
4–6	370.9	3368.5	596.9	2202.5	451.9	2586.0
6–8	534.7	4670.3	1185.5	4463.8	690.3	3945.6
8–10	664.7	5587.0	1648.0	6222.5	864.1	4945.4
10–12	778.0	6380.8	2384.8	8866.8	1056.0	6034.1
12–14	851.0	6876.0	2653.3	9852.5	1193.5	6799.6
14–24	1087.1	8312.5	4140.2	15094.4	1496.1	8351.0
24–30	1166.5	8656.4	4370.1	15887.6	1624.2	8884.4

Table 4
Cumulative excretion (μg) and diastereomeric ratio of *N*,*O*-didemethyltramadol glucuronide (**6**) in urine of three volunteers after oral administration of 100 mg tramadol hydrochloride

Collection interval (h)	Volunteer 1		Volunteer 2		Volunteer 3	
	1*R*,2*R* (μg)	1*S*,2*S* (μg)	1*R*,2*R* (μg)	1*S*,2*S* (μg)	1*R*,2*R* (μg)	1*S*,2*S* (μg)
0–1	n.d.[a]	n.d.	n.d.	n.d.	n.d.	n.d.
1–2	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
2–4	47.1	389.7	58.1	270.0	50.9	309.2
4–6	135.1	1034	121.7	578.5	139.0	1011
6–8	242.5	1724	286.7	1367	243.5	1836
8–10	340.4	2264	434.9	2050	331.7	2422
10–12	446.4	2741	647.0	3017	440.5	3128
12–14	535.1	3093	732.2	3461	515.6	3606
14–24	862.1	4093	1196	6085	734.2	4846
24–30	n.d.	n.d.	1272	6592	817.7	5195

[a] n.d. = not determined.

suitable for the quantification of the tramadol glucuronides requiring only small sample sizes.

Acknowledgements

The synthesis of *O*-demethyltramadol glucuronide by Dr. J.B. Lenfers (Wuppertal, Germany) is gratefully acknowledged. Furthermore, we would like to thank Dr. J. Gerloff (Grünenthal GmbH, Stolberg, Germany) for providing *N*,*N*,*O*-tridemethyltramadol and the volunteers for their helpful cooperation. For financial support we thank the Deutsche Forschungsgemeinschaft and the Fonds der Chemie.

References

[1] R.B. Raffa, E. Friderichs, W. Reimann, R.P. Shank, E.E. Codd, J.L. Vaught, J. Pharmacol. Exp. Ther. 260 (1992) 275.
[2] E. Frankus, F. Friderichs, S.M. Kim, G. Osterloh, Arzneim.-Forsch./Drug Res. 28 (1978) 114.
[3] K. Flick, E. Frankus, E. Friderichs, Arzneim.-Forsch./Drug Res. 28 (1978) 107.
[4] W. Lintz, S. Erlacin, E. Frankus, H. Urrag, Arzneim.-Forsch./Drug Res. 31 (1981) 1932.
[5] B. Elsing, G. Blaschke, J. Chromatogr. 612 (1993) 223.
[6] W.D. Paar, P. Frankus, H.J. Dengler, Clin. Invest. 70 (1992) 708.
[7] P. Overbeck, G. Blaschke, Arch. Pharm. 326 (1993) 682.
[8] M. Bradford, Anal. Biochem. 72 (1976) 248.